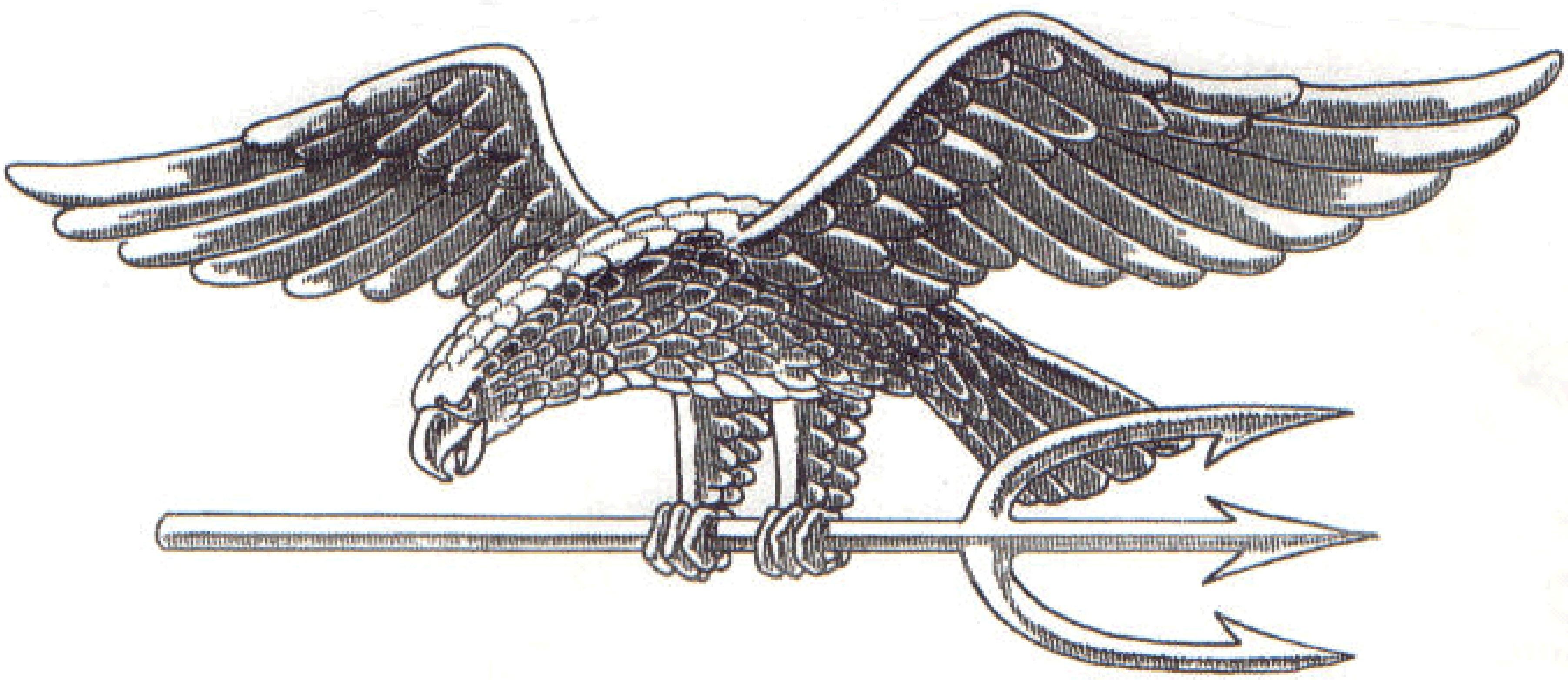
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SCIENTIFIC PRINCIPLES OF IMPROVISED WARFARE AND HOME DEFENSE
Volume 6-1

Advanced Biological Weapons Design and Manufacture

- Biological Entry
- Bacteria Based Weapons



Why pay to recruit troops and build factories to

wage war and kill for you when nature will do it for free

Or, if you can make jello, you can wipe out cities - Enjoy!

For Academic Purposes Only

Advanced Biological Weapons Design and Manufacture

Table of Contents

Advanced Biological Weapons Design and Manufacture

Chapter 1

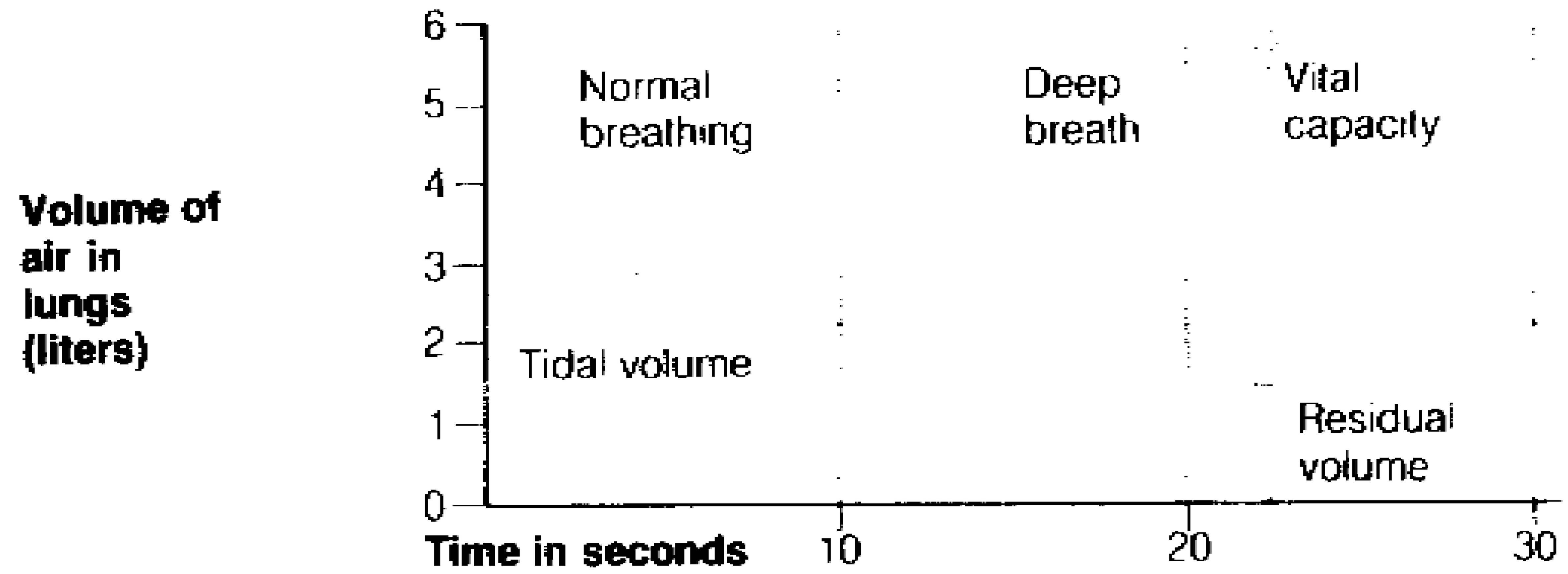
Biological Entry by Respiratory, Gastrointestinal, Genitourinary, and Dermal Routes

In order for any biological (or chemical-see Volume 5) agent to cause harm, it must first be capable of entering the body. The science of how disease and other biologically produced agents can enter the body and cause harm will be covered in this chapter as listed.

A) Respiratory

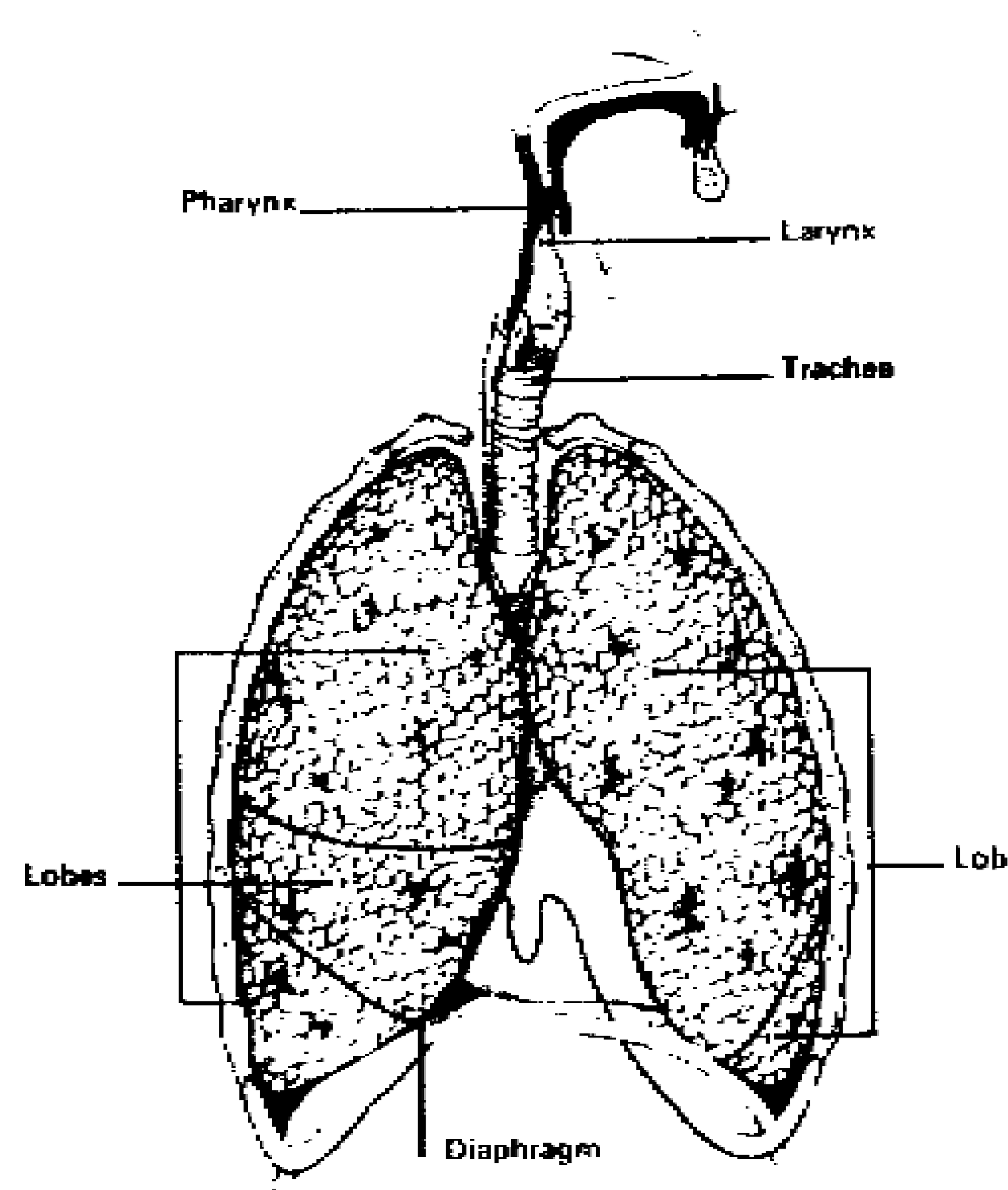
We inhale and exhale 5,000 to 15,000 gallons of air each day.

We take in about 3 breaths every 10 seconds of 2 to 3.3 liters of air while at rest and up to 6 liters when exercising.

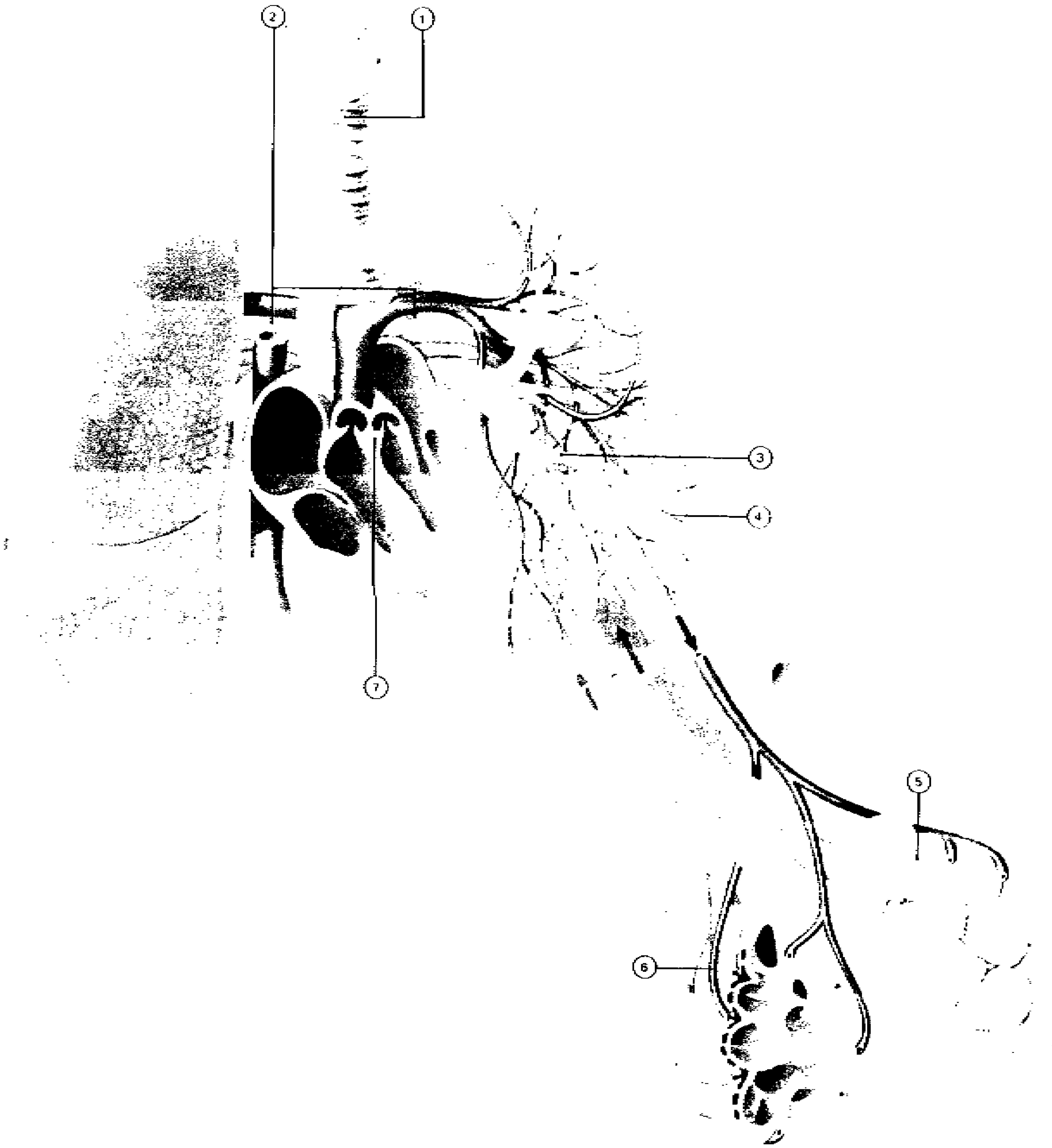


This air usually contains 1,000 to 100,000 particles of dust most of which are too small to be seen. It also contains between 10,000 and 1,000,000 microorganisms. When we breathe and eat, millions of organism come in contact with our mouths and are drawn into the pharynx, past the larynx (voice box), down the trachea, and into the lungs.

As we breathe in, the diaphragm muscles tighten causing it to contract from a dome to a saucer shape. Simultaneously, our rib cage swings upward and outward. As the chest expands, the lungs also expand causing a low pressure area to form. Air rushes down our trachea and into the lobes to restore or balance the normal air pressure on the outside of our bodies with the inside of the lungs. As the diaphragm relaxes the rib cage and thorax return to their resting shape compressing the air in the lungs and forcing some of it out through the trachea.



Air is funneled into the flexible windpipe, our trachea (1), which is ringed with cartilage. Adult trachea are about 10 inches long and branch into the left and right bronchi (2). These branch into several smaller bronchi (3) which continue to branch into more than 250,000 respiratory bronchioles (4) that are each about .02 inches in diameter. The air then travels into the hollow alveoli (5) which are combined with capillaries only one cell width wide (6). Stale blood is pumped from all parts of the body to these alveoli which secrete a thin film of liquid with low surface tension forming a membrane over which oxygen from the air diffuses into the blood. This reoxygenated blood is drawn by pumping action back to the heart (7) to be pumped to all other living cells in the body.



The linings of the alveoli total some 750 square feet in an adult, or more than 40 times the total outside surface area of the body. The right lung consists of three lobes and the left lung has two lobes making room for the heart. The air is filtered by the mouth and the hairs of the nasal cavity which help retain moisture and catch many dust particles and microorganisms. Tiny hairs line the bronchi and bronchioles called cilia. These slowly waft mucus full of cell debris and foreign particles upward to the throat where it is then swallowed and disposed of through the stomach or spit out. Larger dust particles and microorganisms cause the lining to produce extra mucus to catch the foreign matter and these form detritus that must be coughed up and or sneezed out to clear the air passages. When people are ill with infections in the lungs, droplets filled with the disease organisms are disseminated as aerosols which infect other people who breathe them in. This is why using a hand, mask, or handkerchief becomes an important means of limiting spread of airborne agents.

In the lower respiratory tract, the sensitive and delicate alveoli are kept clean of dust particles and bacteria by an army of phagocytes, scavenger white blood cells that engulf and devour the foreign matter.

Many microorganisms that can establish infections in the upper respiratory tract usually are not able to infect the lower tract. Likewise, other organisms can move past the cilia and mucus secretions and infect the lower areas without infecting the upper tissues. Almost all microorganisms are caught and worked out of the upper tract. Those that make it through to the lower tract are consumed by the white blood cells. If the phagocytes are overwhelmed then the infectious organisms can injure the tissues and enter the bloodstream where the infection can spread to all parts of the body.

Some dust particles such as asbestos and fiberglass are caught and tangled up in the cilia and mucous membranes and eventually work there way down into the lower respiratory tract where they accumulate. They are composed of silica and other minerals that the white blood cells cannot break down chemically and eventually cause disease, injury, and suffocation as a result. These types of materials make excellent carriers for biological weapons when ground down to fine particle sizes (400 mesh or smaller). These types of dusts are also excellent carrier agents for toxins and chemicals that inhibit the response of white blood cells, injure tissues, or cause the suffocating exudation of large amounts of mucus which cannot all be coughed out.

The various candidates for add on agents include -

1. Lung Irritants to cause formation of mucous and/or coughing. These are useful when using anaerobic bacteria to cut off oxygen allowing them to reproduce in the lungs, or to cause the coughing up of particles saturated with Botulinum toxin so it is swallowed and quickly absorbed through the intestinal tract and disguising its source of transmission.

2. Lung Injurants which cause internal damage and expose the bloodstream to the organisms on the dust. This allows for rapid infection and spread to other tissues.

3. Bioacting chemicals such as blood clotting agents thrombin and fibrinogen which cause the blood to clot at the site of the dust particle. This allows the flow of blood and oxygen to stop and provides a perfect medium for deadly anaerobic organisms like Clostridium Tetanus, Perfringens, and Botulinum to flourish and mass produce their deadly toxins. Once the clot is broken by expanding gas, the toxins and bacteria spread throughout the lungs and body.

Water soluble toxins produced by living organisms that are breathed in can also be easily absorbed across the air-blood membrane and into the blood stream if they are not breathed out, physically stopped, or broken down as foreign material. This allows production of many war toxins to be designed for use as aerosols to be delivered into the target respiratory tracts dissolved in the breathed in water droplets. This water is conserved and often absorbed by the body with the dissolved toxins which then cause their harm.

As mentioned earlier, a typical cubic yard of our atmosphere can contain hundreds of thousands of bacteria, viruses, fungal spores, pollen grains, lichens, algae, and protozoa. When we are ill with the flu or a common cold, a simple sneeze can fill the air with tens of millions of infective microorganisms. Most of these germs we expel are bound up in mucosy droplets. Most of these are large gobs over four thousandths of an inch across and fall quickly to the floor in a few seconds. If someone else happens to breathe them in, the nose hairs and cilia will usually filter out most if not all of them. Some of the smaller droplets dry and shrink up to "droplet nuclei" only two ten thousandths of an inch across and carry a few bacteria or hundreds of viruses. These colonies can float around for hours or even days on the tiniest currents. The clumped germs help to maintain their critical temperature and moisture and protect themselves from damaging sunlight. They are small enough to easily pass through the respiratory passages until they find a spot to land deep in the tissues.

To give a comparison of the tiny sizes of these microorganisms we will start with fungal spores. These are small and light enough to be picked up by breezes over mushrooms on land in Africa and other continents and be found in the air in the middle of the Pacific and Atlantic oceans. Fungi have traveled on the winds from Mexico to Canada wiping out corn and wheat crops along the way. Rising thermals and gusts of wind picking up the fungal spores and carrying millions of them up to altitudes as high as ten thousand feet in the air. At this height, smooth, interstate like airstreams will carry them along at speeds of up to 40 miles an hour until the rain washes them back to earth and cleanses the air of almost all its airborne dust particles and microorganisms (until they dry up on the ground and are again caught up into the air flows).

The fungi described above are big and sturdy. The bacteria are 100 to 300 times smaller than the fungi. The viruses are 100 times smaller than most of the bacteria. Once all these microorganisms are in they air, they float around until they die off from damage due to sunlight, being eaten themselves, physical damaged beyond repair, or finally land on something that they like to eat. This is mostly the rotting wood, plant tissue, and animal carcasses we see along our way. Sometimes the bacteria land on or in humans and have the right biology to consume our tissues. These organisms make the best weapons.

One of the easiest ways to see this process at work is set out a wet piece of bread on a piece of paper and watch the microorganisms from the air settle on it and quickly produce visible colonies. Some of these molds that settle on the bread produce toxins that can kill human beings in tiny amounts. Some, like penicillium can produce life saving antibiotics. In this book we will teach how to grow and identify harmful microorganisms and describe how they are mass produced and converted into deadly weapons.

On the defense side of protecting ourselves from deadly disease weapons are -

1. The use of physical barriers. These include dust masks, gas masks, filtered air systems used in hospitals, meat processing plants, and military vehicles and protective suits.

2. Use of quarantine to keep infected persons isolated and away from spreading disease into the general population.

3. Use of high concentrations of ultraviolet light to kill microorganisms that might be able to pass through filtered air or to clean the air in suspect rooms and vehicles.

4. Use of chlorinated sprays that are emitted in tiny droplets simulating rain to cleanse the air and to kill any organisms they wash into the ground.

5. Use of early warning systems developed by the US military to detect microorganisms in the air. These devices include -

A pulsing infrared laser system that bolts onto a blackhawk helicopter and transmits pulses up to 20 miles away and analyzes the photons that bounce back. Unusual shaped clouds can often be distinguished as being man made rather than produced by nature when the background readings have been established.

An ultraviolet laser that makes biological particles fluoresce and can analyze clouds up to two miles away. It can distinguish between clouds of dust and incoming plagues.

Air sampling in which lasers are used to identify molecules of life by their adenosine triphosphate content. These are then tested with a battery of antibodies and other compounds to help the military identify what they may be dealing with.

The idea of all these methods is to quickly spot an enemy or terrorist who is spreading the bioweapon and then use military force to stop them. The ease with which these organisms can be spread and their innate invisibility makes it nearly impossible to stop them from being used.

Air conditioning systems use powerful vents to sweep air over water and these can pick up toxic clouds of droplets and carry them throughout buildings. This is how Legionnaires disease first sprang upon mankind. Supermarket vegetable misters, whirlpools in spas, decorative fountains in public gathering areas, and even hotel showers can be quickly converted into biological weaponry by respiratory routes. Even passing through guarded gates is possible by "conscripting" the general public and key employees to carry the microorganisms under the body of their cars. These can be releases as the hot exhaust melts container coverings and release aerosol and dust particles and distributes them into the air. If these are detected, the unwitting conscriptee ends up being unjustly arrested and accused, tying up valuable enemy resources, creating mistrust and anger, and creating false trails.

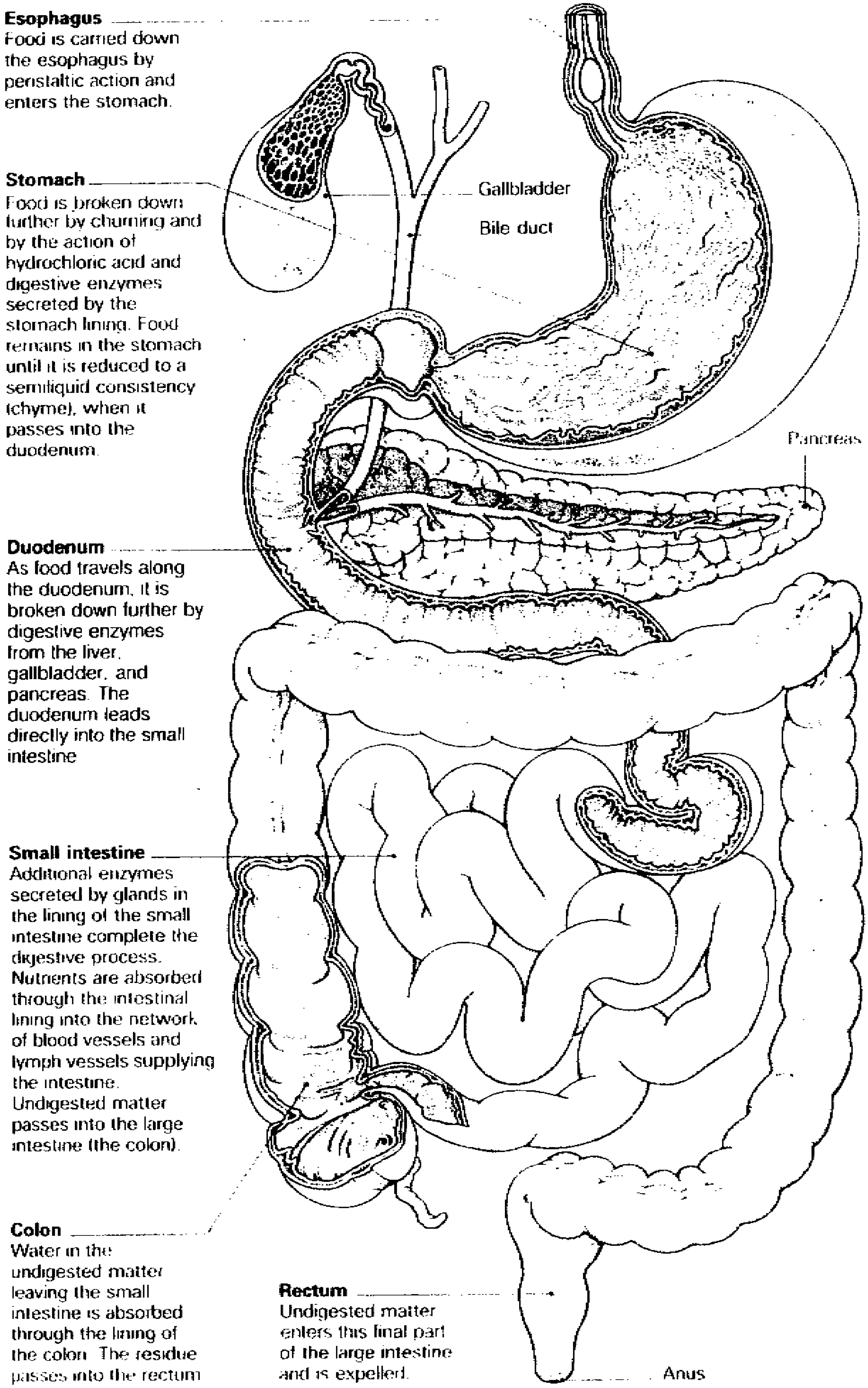
B) Gastrointestinal

All substances that enter the mouth can be expelled or breathed back out, proceed down the respiratory tract with the air, or be swallowed, usually by being mixed into saliva and various solid boluses which are then forced to the back of the mouth by the tongue and then propelled down the esophagus.

In the digestive tract, foods are broken down into their basic biological components that can be then absorbed into the bloodstream and used by our cells. Saliva in the mouth begins this process. The glands of the stomach lining produces two to four pints of gastric juices each day. This juice is composed of strong hydrochloric acid which kills almost all microorganisms, protective mucous, and the digestive enzyme pepsin. This combination and the actions of the stomach muscles reduces food to a pulp in about an hour.

From here it goes into the first curved sections of the small intestine which is about 23 feet long in most adults. Most digestion and absorption of nutrients takes place here as the small biological components easily go into solution and pass the membranes of the intestinal lining and into the surrounding bloodstreams.

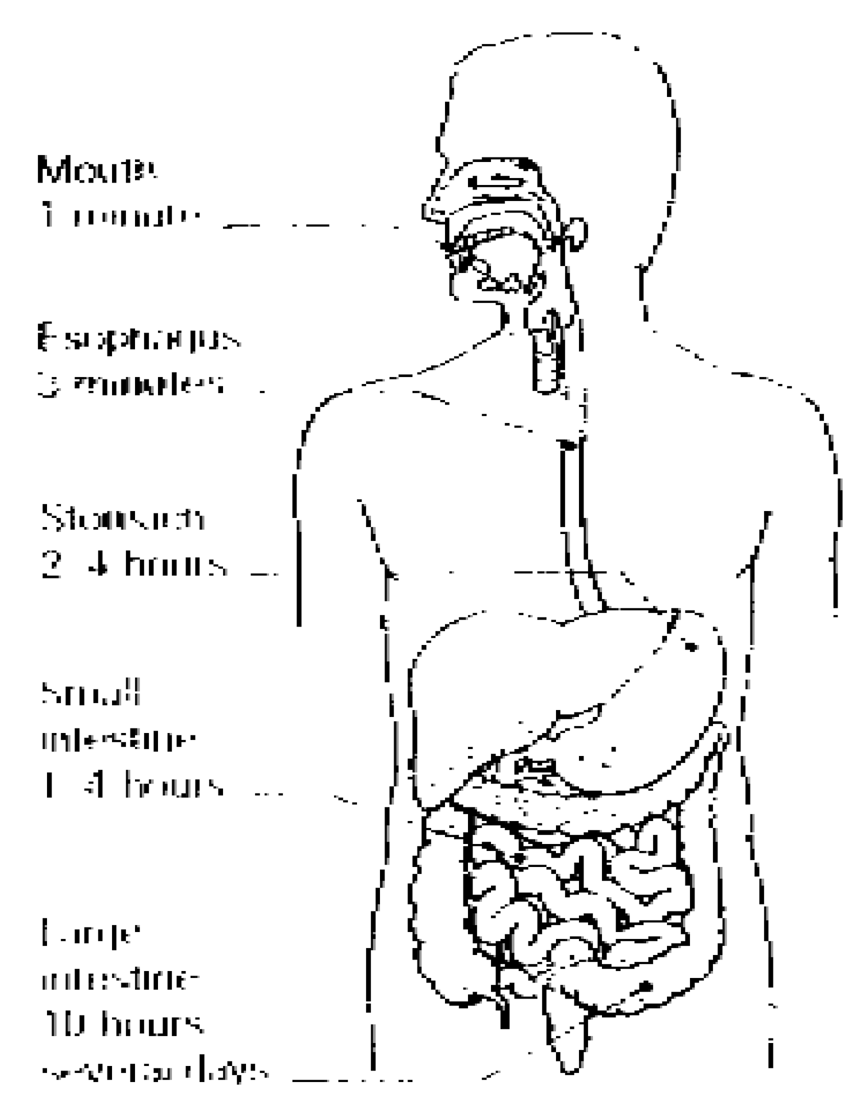
In the small intestine pH changes from acid to alkaline as the new gastric juices from the pancreas and the gall bladder pour into the duodenum. These juices include bile salts, a thick green bitter liquid formed by the liver and stored in the gall bladder, which provide a measure of protection against invading gram positive bacteria that survived the stomach acids by killing or stopping virtually all growth of this bacterial group. Many selective culture media use bile salts extracted from dead animal tissue in slaughter houses to inhibit growth on culture plates and slants which then allows only gram negative bacteria to grow. Using this green liquid "squeezed" out of the gall bladder tissue and incorporated onto sugar free Jell-O media allows the field improvisation of culture plates used to isolate prospective biologicals for use as weapons. This is important because these types of organisms are ideally suited to live and reproduce in the human intestinal tract and make excellent weapons. Cultures can use bile salts to weed out all the other contaminating strains.


Esophagus
Food is carried down the esophagus by peristaltic action and enters the stomach.
Gallbladder
Bile duct
Stomach
Food is broken down further by churning and by the action of hydrochloric acid and digestive enzymes secreted by the stomach lining. Food remains in the stomach until it is reduced to a semiliquid consistency (chyme), when it passes into the duodenum.
Pancreas
Duodenum
As food travels along the duodenum, it is broken down further by digestive enzymes from the liver, gallbladder, and pancreas. The duodenum leads directly into the small intestine
Small intestine
Additional enzymes secreted by glands in the lining of the small intestine complete the digestive process. Nutrients are absorbed through the intestinal lining into the network of blood vessels and lymph vessels supplying the intestine. Undigested matter passes into the large intestine (the colon).
Colon
Water in the undigested matter leaving the small intestine is absorbed through the lining of the colon. The residue passes into the rectum
Rectum
Undigested matter enters this final part of the large intestine and is expelled.
Anus

The lining of the small intestine is heavily folded to increase its surface area using thousands of minute fingerlike projections called villi which waft back and forth bringing the mix of foods into contact with the blood capillaries and lymphatic vessels that are part of each villus. They then diffuse into the these structures which transport them throughout the body. Toxic water soluble substances produced by biological organisms that were not broken down by the gastric juices will enter the blood and lymphatic systems at this point.

The undigested portions of matter which includes cellulose, gastric secretions and other debris then pass through the ileocaecal valve into the caecum, a small pouch at the start of the colon, the main part of the large intestine. Water and other important chemicals are retrieved and reabsorbed into the bloodstream here. The final waste material is then disposed of by the colon in the form of feces.

Microorganisms enter the intestinal tract continuously in huge numbers. Some of these live naturally in the oral cavity, nasal passages, and tissue linings. Most are living in or on ingested food and water. In the digestive tract are large populations of healthy bacteria that help maintain good health by competing with and crowding out disease causing organisms that survive the stomach juices. These include lactobacillus which excrete small amounts of lactic acid and this generally inhibits growth of many dangerous bacteria.



The two main processes which initiate disease and harm in the intestinal tract are -

1. **Food poisoning or intoxication**. This occurs when a living organism such as a poisonous death cap mushroom produces a deadly toxin, or clostridium Botulinum yields its deadly botulism protein. These are then eaten and the toxins are absorbed into the bloodstream with other nutrients and from there travel into the body into locations where they cause harm. These can be neurotoxins which travel to the central nervous tissues or toxins which inflame and injure all tissues they come in contact with.

These types of weapons can be mass produced from the originating microorganisms on culture media and then mass produced, harvested, and finally incorporated into the delivery system which can be used to poison foods, water, eating utensils, condiments, handling surfaces such as restroom door handles and air dryers and so on. They can also be incorporated into inhalation, dust, or aerosol weapons with irritants that cause them to be coughed up and swallowed into the intestinal tract.

2. **Invasive pathogens** survive the gastric juices and make their way into tissues they can live on and harm. Most of these requires very large numbers of organisms to both survive the stomach acids and the competing bacteria before establishing an infection. Usually this requires from 100,000 to many millions of organisms being swallowed, depending on the type and form (such as spores) of organism used.

Anthrax and Clostridium spores can often survive the acids and other bacteria. The clostridium require little or no free oxygen to reproduce and are not normally a problem as the intestinal tract contains too much oxygen for them to survive and reproduce. The anthrax can survive and reproduce and is very deadly when eaten.

E Coli, Salmonella, Shigella, and Cholera are examples of infective gram negative bacteria that can easily establish infections and harm human hosts. These will all be described in detail in the next chapter.

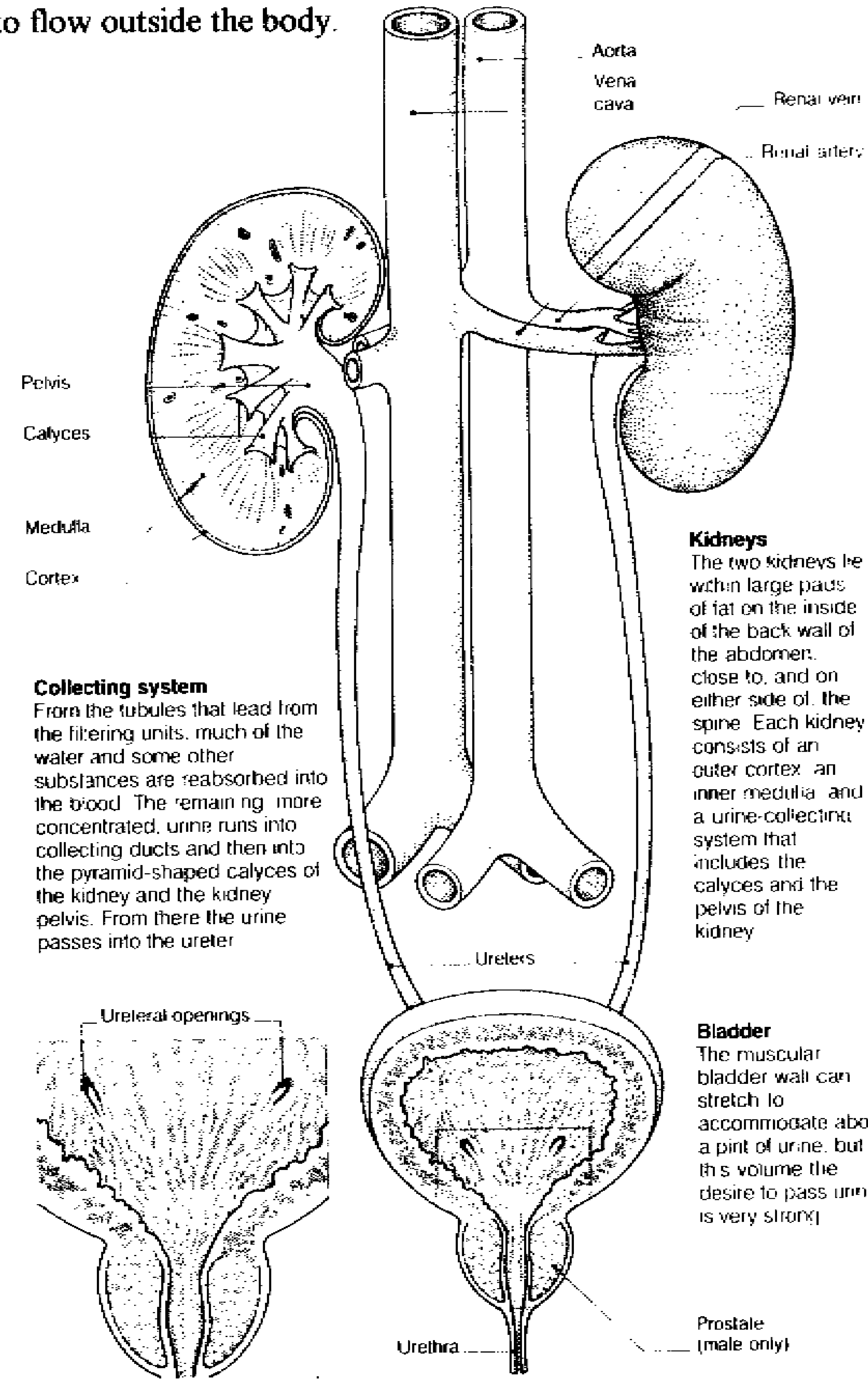
C) Genitourinary

The genitourinary system consists of

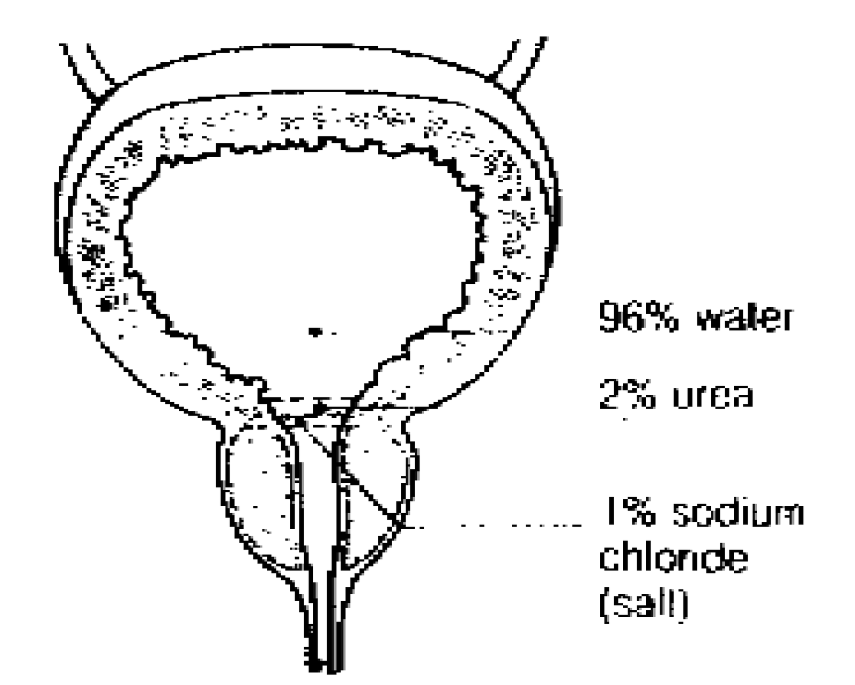
A pair of dark red, bean shaped kidneys 4" long and 2" wide weighing about 5 oz.

A pair of ureters, muscular tubes 10-12 inches long carrying urine to the bladder

A hollow muscular bag forming an app. 1 pint reservoir of urine. The sphincter is a muscular ring at the exit that contracts to hold urine in. When relaxed it releases liquid allowing the urine to flow to the urethra which is a muscular tube which allows the urine to flow outside the body.



COMPOSITION OF URINE
Urine consists mainly of water, with small amounts of urea (the main waste product), other waste products, and salt.



Interior of bladder
The two ureteral openings and the urethral orifice form a triangle on the base of the bladder. In males, the urethra runs through the body of the prostate gland situated below the bladder

Two types of disease processes are useful in producing weapons of the genitourinary tract.

1. **Opportunistic infections** which contaminate the GU tract colonizing and harming tissues in the genital areas and working their way into the bladder and kidneys. The bacteria Proteus has the ability to break down urine into ammonia and carbon dioxide and when it infects the bladder and kidneys it can be quickly life threatening. Toxic shock syndrome is caused by Staphylococcus aureus which produces deadly toxins that enter the bloodstream during menstruation.

2. **Sexually transmitted diseases** which usually enter the body through abrasions during sex.. These include Gonorrhea, Syphilis, and AIDS. These types of diseases can be grown in culture and incorporated into abrasion dusts, ie. weapons that cause scratching like poison ivy extract and itching powder. Once the itching begins the abrasions are loaded with the disease organisms at the contact points and under the fingernails of the targets. Once in the tissues, infections begin in many locations.

AIDS is the one weapon that has been effective in its natural state. As long as it is maintained in healthy human blood, it can be passed to others by mechanical means as has been demonstrated with needle sharing of drug addicts and blood transfusions. Mechanical methods may include the use of pungi stick type weapons incorporating reservoirs of infected blood or a blood soaked dust weapon described above.

One final potential weapon has been observed on videotape and used in prosecutions. Instances where an individual urinates into someone's coffee or other drink can possibly poison directly by the presence of urea. It can very quickly kill if the urine is cloudy due to infection. The buildup of huge numbers of organisms in colonies (in the billions for each colony) that can actually be seen, and the accumulation of toxins can cause very rapid infection and or death.

D) Animal and Insect Bites

Many animal and insects act as reservoirs or **vectors** for a wide range of disease organisms that harm people. The vectors transmit the organisms usually by biting a person and passing the microbial into the tissues with saliva or other liquid. The range of disease is wide and includes viruses, bacteria, parasites and so on. Some of the deadliest diseases known to man are disseminated to human populations in this manner. These include -

Rabies
Yellow Fever
Dengue Fever
Encephalitis
Tick Fever
Plague
Tularemia
Rocky Mountain Spotted Fever
Typhus Fever
Chagas
Sleeping Sickness
Malaria

Transmitted by ticks, mites, mosquito's, lice, fleas, and other small insects and animals, these are capable of very large scale harm. Each of these will be covered in detail in later chapters.

Two main characteristics for these weapons are

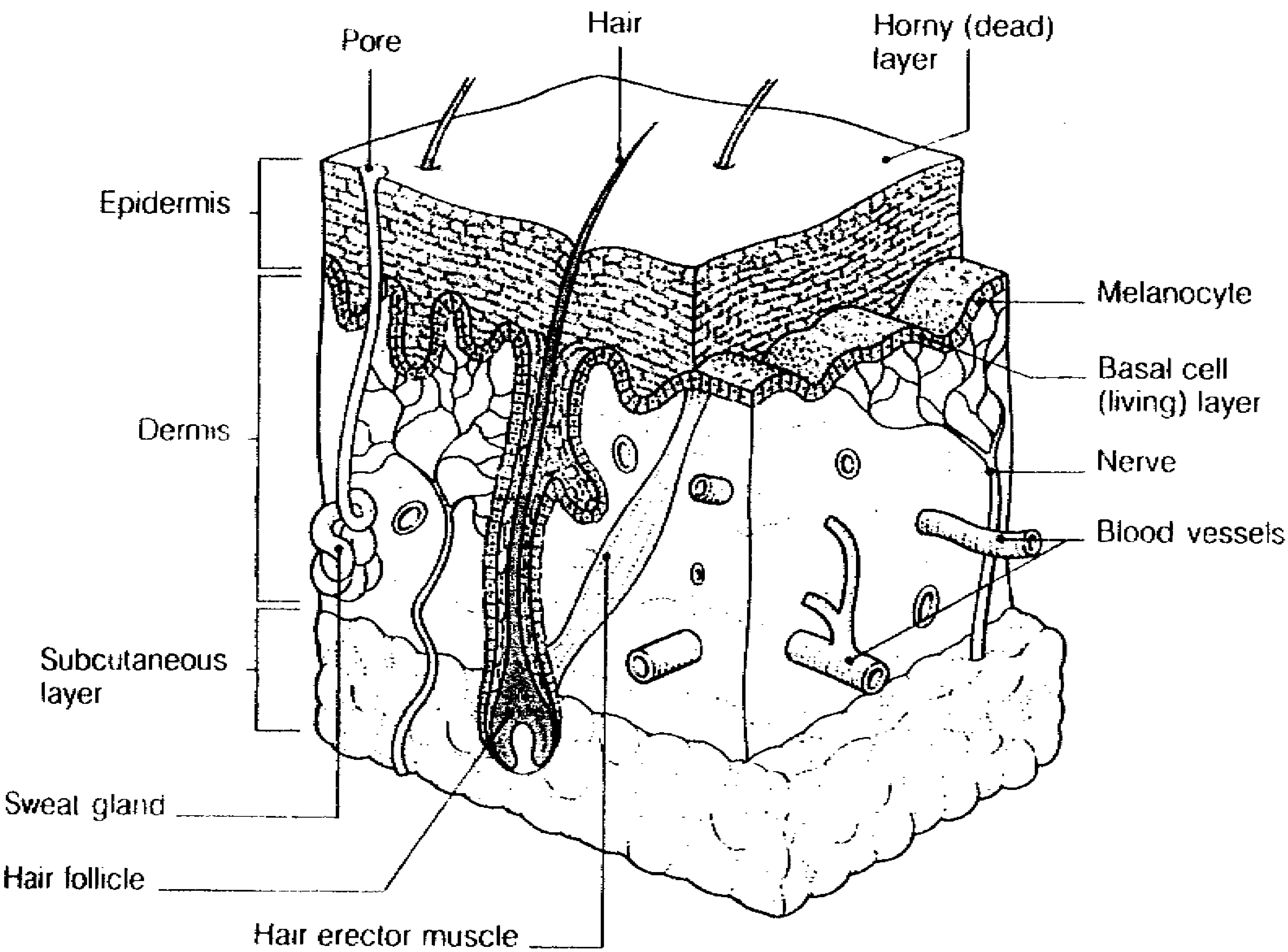
1. They do not survive long outside humans or the host carriers. This requires special media to keep them alive for use in weapons.

2. They require a way of surviving entry into the body so they can infect the target tissues. In nature this usually entails puncturing the skin which requires the use of piercing weapons or irritant carrier dusts to create entry portals and aid in mechanical deposit and distribution. Many of the organisms can be transmitted via the respiratory tract if they can survive the conditions of transport and delivery.

E) Direct contact from wounds, burns, chemical injuries or abrasions

Many biologicals can harm or kill by passing through the skin or colonizing it and producing deadly toxins that are then absorbed into the bloodstream

. The skin is made up of two main parts, the "Epidermis" is the outside protective layer which contains disposable dead cells for its outer layer or covering. The "dermis" (true skin) lies underneath and contains most of the living elements. Below the skin layer lies the main body tissues and circulatory system which moves all the nutrients around.



The skin is the largest organ of the body and protects the internal organs from the environment. The skin loses dead cells constantly to wear and tear and these are continually being replaced as new skin grows underneath.

The outer layer Epidermis is made up of flat cells that resemble paving stones when examined under a microscope. It is thickest on the body parts that are used the most in contact with the solids in the environment, such as the soles of the hands and feet. It is very thin in the eyelids and allow some light to pass and is generally thinner in women than men. The skin also thins with age.

The outer part of the epidermis is made up of the dead cells that form the tough, horny, protective coating. As these cells are worn away they are replaced by rapidly dividing living cells in the innermost part of the epidermis. In between is a transition region that is made up of both living and dead cells. Some of the cells produce the protective pigment melanin which gives us our skin color. At the base the cells grow flatter and fill up with keratin (which also makes hair and nails) and die. The dead cells form in stacks about 30 cells thick.

The Dermis is made up of connective tissue mixed into other specialized structures like the hair follicles, sweat glands, and the sebaceous glands that produce an oily substance called sebum which is made up of fats and wax. It lubricates the skin, keeps it supple, and prevents it from becoming sodden when we are swimming or bathing, and keeps it from drying out in dry climates. It also helps seal the skin surfaces to keep out bacteria, viruses, fungi, and tiny particulates. The dermis also contains blood and lymph vessels and nerves.

The skins most important function is to protect us by shielding our tissues from abrasion, harmful sunlight, invasion from harmful organisms, and chemicals. The skin is also a sensory organ and contains many cells that are sensitive to pain, pressure, and itching. It also helps keep our body temperature constant. When we are hot the sweat glands release water that cools our bodies down, and the blood vessels in the dermis dilate to help let the air cool the blood that is circulating. When we get cold, the skin constricts to hold in heat. Sweat glands close up and the hairs grow erect to produce a layer of still air that insulates the skin.

The epidermis contains enough sebum to make our skin waterproof so that we can swim or bathe without soaking up water like a sponge. The outer epidermis also can hold some water which helps make it elastic. If this water content drops below a certain level our skin cracks, which reduces its efficiency as a barrier. Our bodies are made up of 60% water and its stops most of it from evaporating away.

The skin can be injured through cuts, bites, radiation from the sun or other sources, burns, and chemicals. The skin also releases an antiseptic liquid from glands that help to heal wounds. The skin is usually about 1/16th of an inch thick but can be 1/8th" thick on foot soles and palms. As the body ages, the body bulk shrinks and the skin loses its elasticity causing bagginess and wrinkles.

Biological agents must have means of entering the skin tissue to cause harm. These methods involve -

1. Abrasions caused by itching producing irritants, allergic reactions to various substances, physical injuries resulting in scratches or scrapes, and those produced by deliberate mechanical means such as punctures from pungi sticks, knives, and bullets.

2. Burns from acids, alkalis, sun or ultraviolet light, or heat such as flame and hot surfaces. These cause physical destruction of the protective layers of skin cells and allows the microorganisms to directly contact the moist and nutrient rich tissues and blood vessels underneath.

3. Direct attack by organisms such as infective fungi, viruses (warts, smallpox), bacteria (acne, leprosy), and so on.

The most dangerous is when portions of the body are wounded allowing direct invasion of enormous numbers and types of microbes. The most deadly and common are the Clostridium species Tetanus and Perfringens (gas gangrene), Staph, Strep, and Pseudomonas, and various fungi. These rapidly multiply, producing gases that expand and further damage surrounding tissues, and produce toxins that are carried into the body by diffusing into the adjacent blood vessels. These accumulate until they produce lethal effects.

Chapter 2

Bacteria Based Weapons

Bacteria exist as a tiny group of single cell organisms that can only be seen under the most powerful lenses of a microscope. As a group they have caused the greatest combined loss of life in the history of man dwarfing all the combined wars of all of history. Because of their ability to grow on or in not only human bodies but artificial food sources and produce harmful substances, they are among the most ideal weapons of war.

In this chapter we will describe -

1. How to grow bacteria and how to use language to describe and tell the bacteria species apart.

2. How to make culture media to grow and separate strains of bacteria and how to mass produce desired species.

3. Each potential war weapon species will then be described in detail -

A) History of the bacteria and where and how it can be located and collected.

B) How it can be selectively separated, cultured, and produced.

C) How its cells and toxins can be mass produced and purified.

D) How antibiotics, vaccines, and natural immunity is used in protection against these diseases.

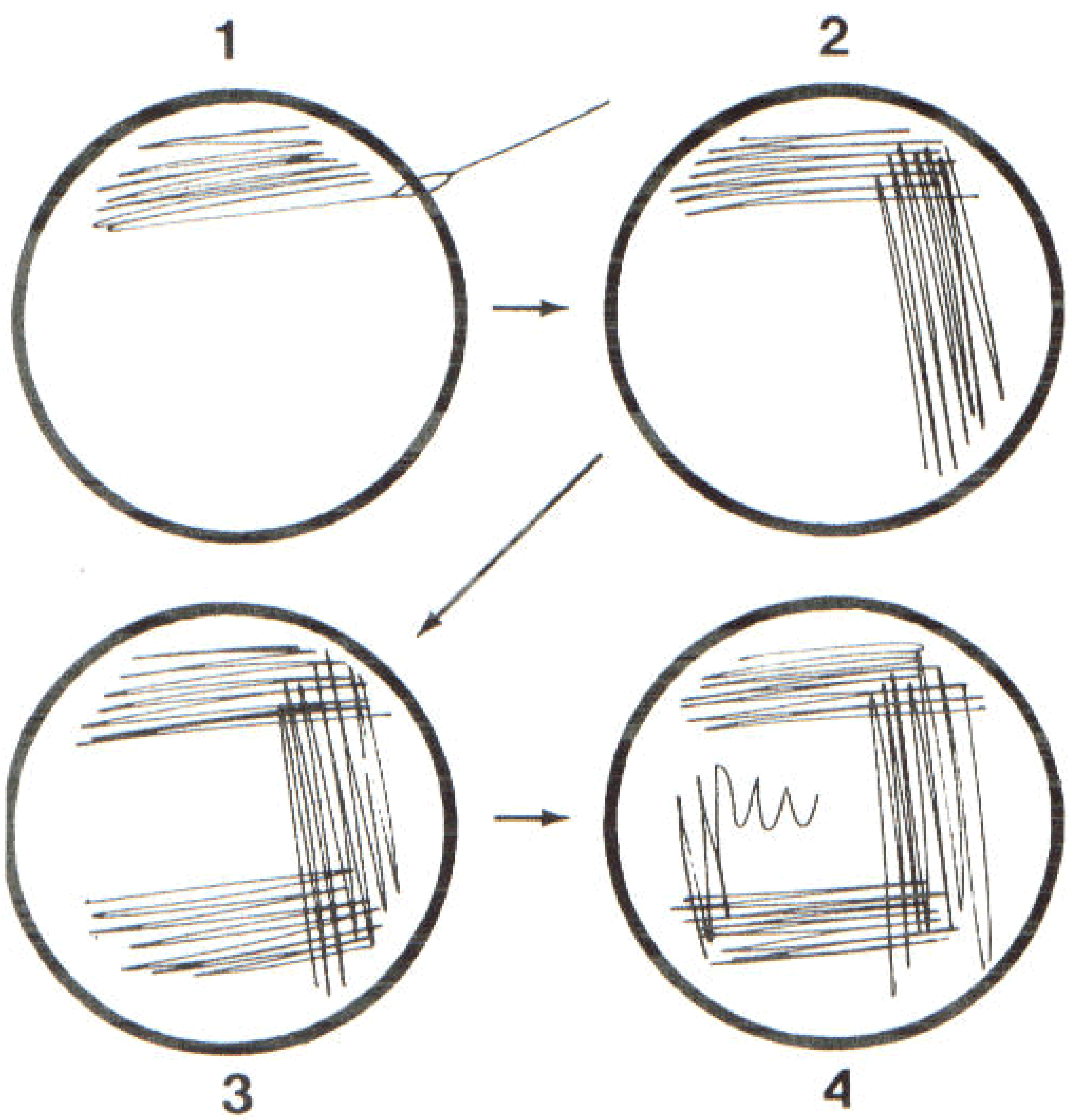
E) How these materials can be incorporated into effective weapons of war.

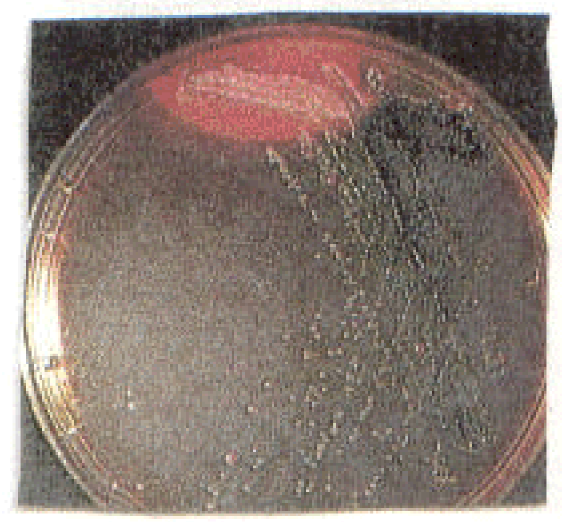
5. Odors sometimes aid in identification of some species -

Pseudomona species	(grape juice)
Proteus species	(burned chocolate)
Streptomyces species	(musty basement)
Clostridium species	(fecal, putrid)

6. Microscopic examination which differentiates by gram stains, spores, external organs and so on. Their are many books an medical microscopy and this material will not be covered here.

When culture media is inoculated, a specimen, or starting material is swabbed or dabbed with a sterile wire with a loop on the end. This is streaked across the surface of the plate so that the inoculum becomes diluted and the colonies can be well isolated. These individual colonies can then be subcultured to other media allowing production of pure cultures.







How to grow and Identify Bacteria

If you place a piece of bread out on a countertop and sprinkle water on it, in a few hours to a day you will begin to see something growing on it. In the air around us are millions of tiny invisible microorganisms that float around waiting for a chance to land on something that they can eat. In the case of the slice of bread, both food, and the necessary moisture were present to feed a small fungal spore that started to grow on the solid bread surfaces. Once the spore could start eating the moist bread, it split into two parts in about 20 minutes. These two split again in about 20 minutes making four cells and so on. As this continues, the numbers of cells grows enormously until you can finally begin to actually see the huge mass. This doubling of the cell mass every 20 minutes or so produces what we call colonies.

20 minutes	=	2	cells	
40 minutes	=	4		
1 Hour	=	8		and doubling every 20 minutes
2 Hours	=	64		
3 Hours	=	512		
4 Hours	=	4,096		
5 Hours	=	32, 768		
6 Hours	=	262,144		
7 Hours	=	2, 097,152		
8 Hours	=	app	8 Million	
9 Hours			64 Million	
10 Hours			512 Million	
11 Hours			4 Billion	
12 Hours			32 Billion	

At about 12 hours of this kind of doubling, we can begin to see bacteria or molds just starting to become visible in tiny specks to the naked eyes. In one tiny little spot that we call a colony, we can have many billions and trillions of single cells piled up on top of each other growing on the culture medium. In this case it was a slice of wet bread that is ideal for growing molds.

From how these colonies look as they grow and by changing what we feed them we can control what kinds of microorganisms we grow so we can get most of the kinds of bacteria or molds that we want. In addition, we can tell them apart.

It was learned in the early 1800's how to finally grow bacteria on or in artificially prepared mediums. These first mediums were called broth's and comprised finely powdered dusts that were mixed into boiled water. These produced something resembling a thick soup. By varying the different food in the dust, biologists found that they could get different organisms to grow on the top surface of the broth. These microorganisms used the oxygen in the air along with the solids in the broth as their food sources to grow on.

It was soon discovered that some bacteria could only grow underneath the surface in the broth mix and would not grow near the surface if there was oxygen or air present or if dissolved oxygen was in the broth. The bacteria that required oxygen to grow were called **Aerobic** and those that could only grow away from the air or oxygen were called **Anaerobic.**

Other kinds of mediums and methods were soon developed to grow the bacteria and more easily tell them apart. Some of these methods included using egg white (albumin), egg yolk, both parts together, and then mixed with water and other growth nutrients. Many bacteria would not grow satisfactorily using eggs as the basic medium. This led to the use of Gelatin, or what we can now buy as sugar free Jell-O at the grocery store. This worked well for many bacteria when other nutrients like dried milk were added to support their growth. The semi-solid mass would grow colonies with different appearances depending in their species and this made it possible to tell many of them apart by appearance alone. The main problem with using gelatin was that many gram negative bacteria would eat the nutrients mixed in the gelatin and produce wastes that had large amounts of acids mixed into them. These end product acids could be used to tell the bacteria apart, but the acid would break down the gelatin turning it into tiny pools of water around the colony and drowning the colony thereby stopping its growth.

A new substance was soon developed by extracting a polysaccharide from seaweed called agar. Mixed into distilled water at 2%, this material would stand up to almost all pH ranges and temperatures that bacteria would grow and produce on. It never broke down from the bacterial by products and soon replaced gelatin as the new way of producing "jells" to grow bacteria on.

The science of being able to produce a gel medium for growing bacteria is essential in being able to grow bacteria and toxins for use as weapons. The most basic gel mediums and food sources will be described here so anyone can be capable of producing their own microbial growth materials.

To study and observe bacteria colony characteristics, you must produce a solid gel surface on which to inoculate and grow the bacteria. Solidification is achieved by adding agar at 2-3% of the water content, or gelatin at 1-4% (follow the label directions), serum, or egg albumin. These are usually prepared by using distilled water and boiling it to mix in and dissolve the gelling agent. The container must be boiled (or autoclaved if available) to kill off any contaminating bacteria. Distilled water is used so that contaminating minerals such as toxic copper sulfate are not present.

Different food sources are then added to grow the desired group of organisms. These are usually dissolved with the gelling material at the start of the procedure.

The basic starting medium we will use in our example is -

1,000 ml of boiling, distilled water
10 to 20 grams of a basic growth material such as dried milk
20-60 grams of agar, or gelatin

These are mixed together while boiling in a desired container. Once mixed and the materials are all dissolved, additional distilled water (1,000 ml) is added to quickly cool the mix as in the instructions for Jell-O. The mix is then poured into a petri dish (to a depth of about 1/2 inch) if available and a cover placed on it. Other plate type containers may be used. They should be transparent plastic or glass to be used to best effect. The containers with the agar or gel are placed into the refrigerator and allowed to solidify.

In place of the dried milk in the above example, the standard medium for growing disease organisms has become the use of 5-10% sheep or human blood (100-200 grams). Diseases that like to grow on human tissues and blood grow well on this type of mixture and most organisms can be grown on it.

The following materials can be added to the above basic dried milk or blood agar/gel formulas. Per 1,000 ml of water -

Sodium Chloride (Table Salt)	5 grams	Used to increase the range of growth of organisms
Beef Liver	500 grams	To grow brucella
Dextrose	40 grams	To grow Yeast's
Burnt Yeast/ Bread	500 grams	To grow various molds
5-10% Blood heated to 80 C [Heating the blood causes the red cells to lyse and the proteins to precipitate making them more available, hemoglobin is also converted to Hematin]	100-200 grams	To form a "chocolate" agar for growing finicky disease organisms
Potato and Salt	125 grams 5.5 grams	Isolating and growing Bordetella
Phenylethanol (evaporates in 12 hours-use immediately)	2.5 grams	Inhibits most gram negative bacteria, good for growing selectively, gram positive bacteria

Oxgall and Blood	43 grams 7.5%	Inhibits almost all except Streptococcus faecalis
add iron citrate and esculin	.5 gram 1 gram	and it grows group D Streptococci and Listeria
Potassium Tellurite and Blood	1% 5%	Corynebacterium diphtheria - Colony blackening allows for differentiation
Salt d-mannitol Phenol Red	75 grams 10 grams 2.5 ml	Highly selective for Staph from stool specimens. Aureas strain changes media yellow to red
Bile Salts and/or Crystal violet	1.5 gram .001 gram	Inhibits most gram positive organisms. In blood agars, the lactose fermenting gram negative bacteria convert the bile salts color from red to purple/pink
Bile Salts Sodium Citrate Sodium thiosulfate Ferric citrate Brilliant Green Neutral Red Lactose	8.5 grams 8.5 grams 8.5 grams 1 gram .00033 gram .025 gram 10 grams	Highly selective for Salmonella/Shigella citrate and green inhibit most coliforms. Thiosulfate is converted by most salmonella to H2S which reacts with the iron citrate to form blackening around these colonies
Sucrose	5 grams	Some coliforms ferment this sugar more easily
Sodium desoxycholate Ferric ammonium citrate	5 grams 2 grams	Inhibits most gram + bacteria
Bismuth Sulfite Brilliant Green	8 grams .025 gram	Inhibits most gram + and coliform bacteria - good for Salmonella Typhi

Sodium Selenite	4 grams	Toxic to coliforms and aids
at pH of 7 plus		Salmonella growth
Disodium Phosphate	10 grams	Maintains pH at 7 for 12 Hrs

L-Lysine	5 grams	Inhibits gram - bacteria
Burnt Yeast	3 grams	Lactose and sucrose
Xylose	3.75 grams	fermenters produce yellow
Lactose	7.5 grams	colonies, non lactose = red
Sucrose	7.5 grams	H2S production = Black
Sodium desoxycholate	2.5 grams	
Ferric ammonium citrate	.8 grams	
Sodium thiosulfate	6.8 grams	
Sodium chloride	5 grams	
Phenol red	.08 grams	
pH of 7.4		

Phenol Red	.08 grams	Acid production produces yellow colonies Good for clostridium species pH drops also change colony color

Trypticase	40 grams	Good for growing
Salt	10 grams	Clostridium
Sodium thioglycolate	2 grams	
desired sugar (by species)	20%	

Cystine	.5 grams	Good for detecting
Trypticase	20 grams	fermenting abilities of
Sodium chloride	5 grams	Corynebacterium and
Sodium sulfite	.5 grams	Neisseria
Phenol Red	.017 grams	

All enterobacteria use dextrose and produce end acid products.

add	Bromcresol purple	.01 gram	yellow colonies
	Cresol red	.005 grams	green colonies

Thioglycolate	.3 ml	allows anaerobic growth in lower part of the medium

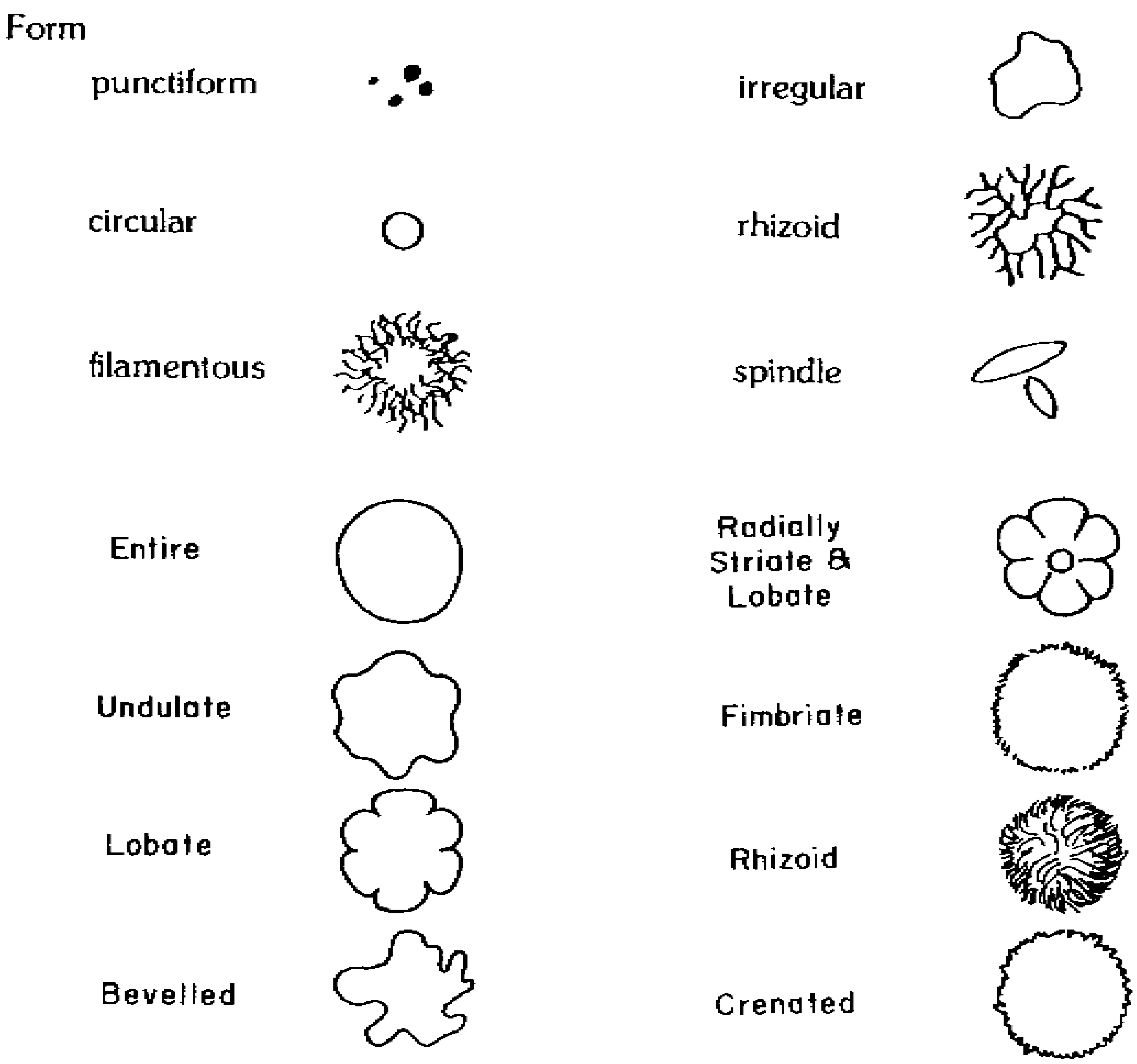
Egg Yolk	10 ml	lecithinase from clostridium turns medium opalescent

Once the medium is prepared and the colonies are growing, they can be identified in part by their growth appearances on the plate. This is called colony morphology. The words used to describe the differences in appearances seem to be different in different textbooks. The two groups of appearance descriptions below will give you a good idea of what these characteristics are called.

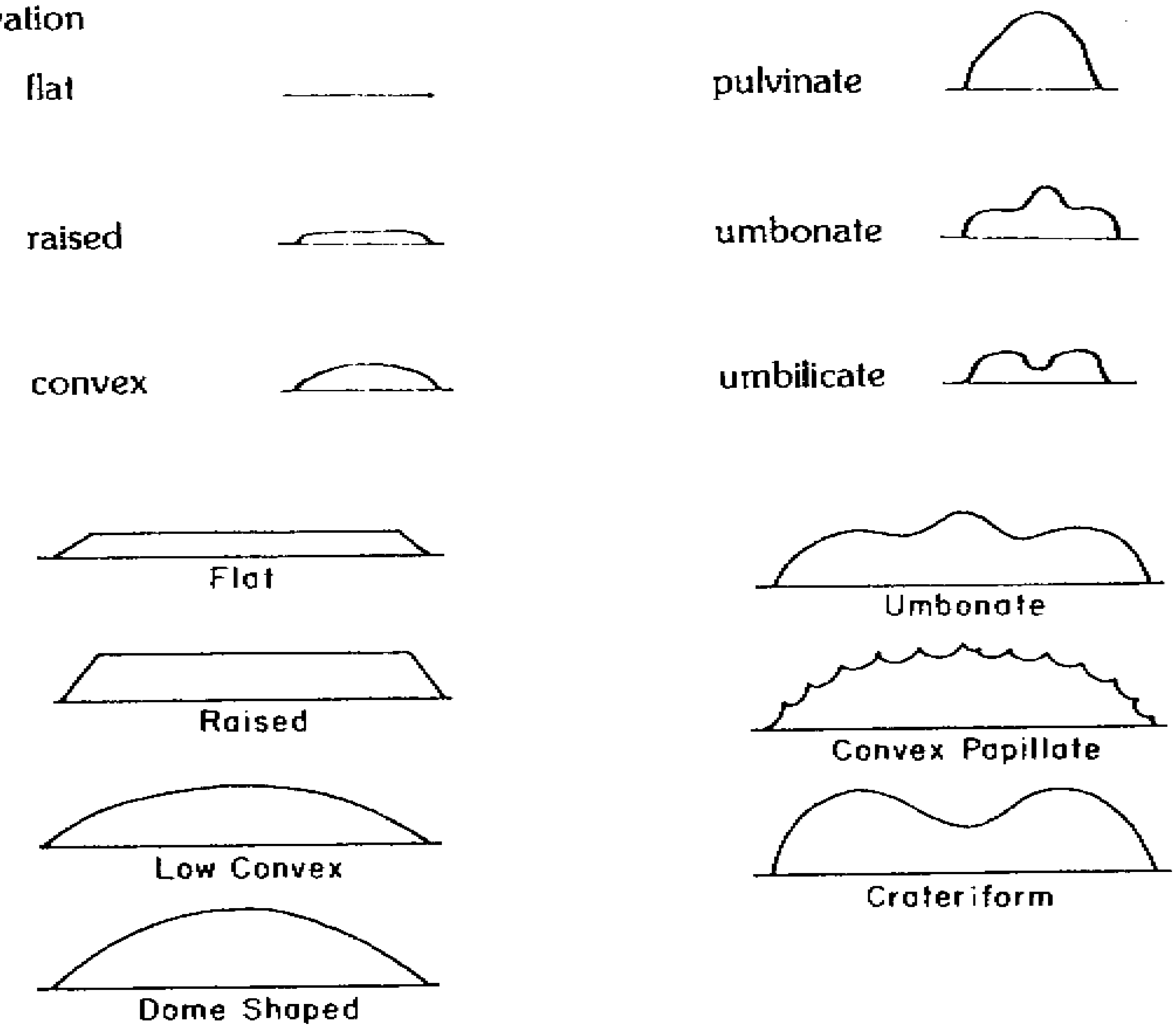
Form is what they look like from the top of the plate looking down.

Elevation is what the colonies look like from the edge.

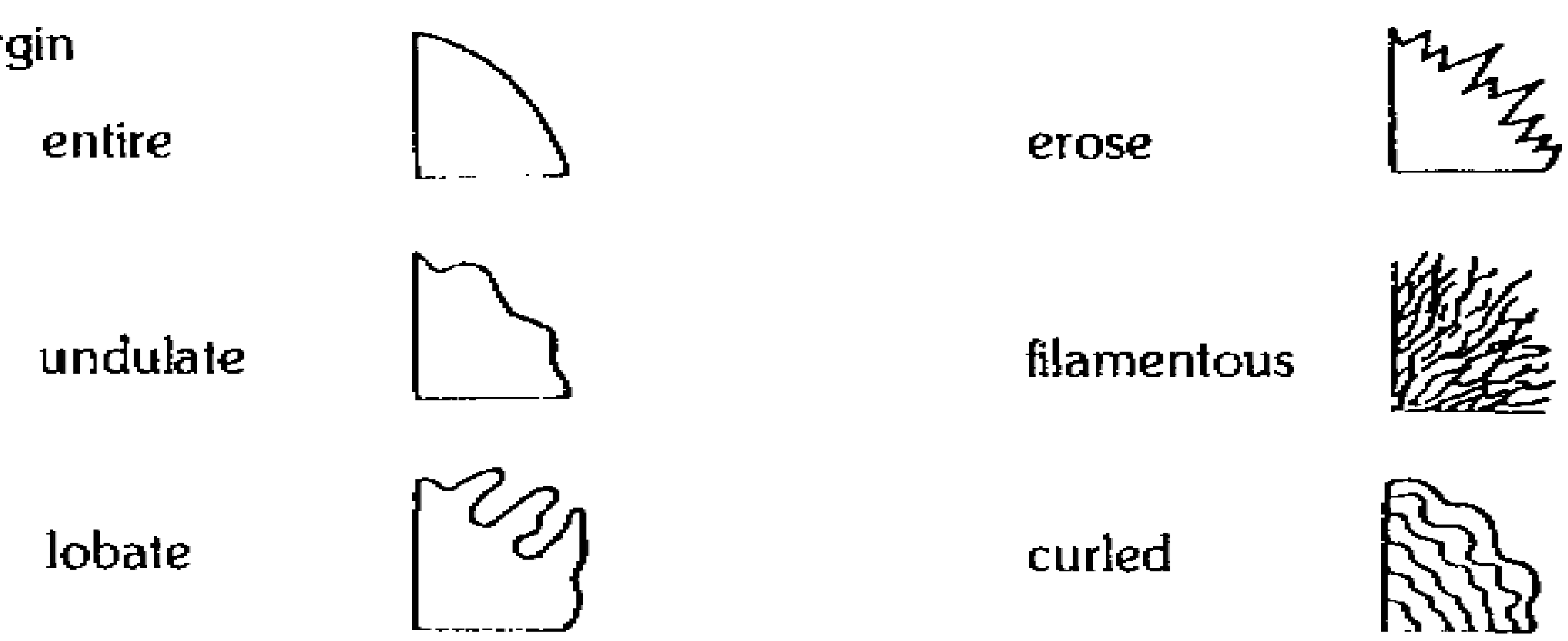
Margin is how the colony grew out along its periphery surfaces.



Elevation



Margin



Colony characteristics used in identification include -

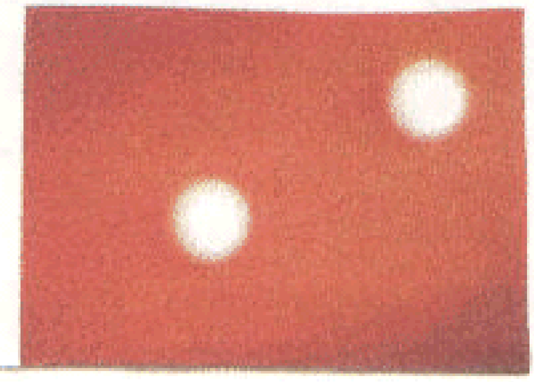
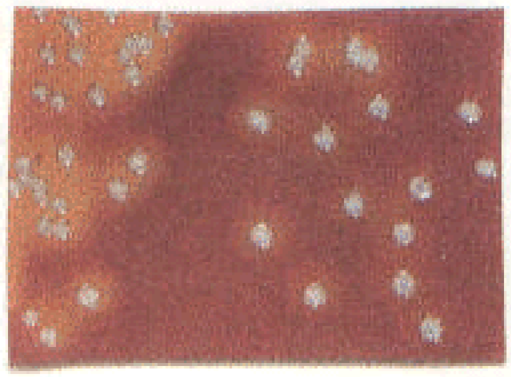
1. Size: Diameter in mm.
2. Form: As listed and illustrated
3. Elevation: As illustrated
4. Margin: As illustrated
5. Color: White, yellow, black, buff, orange, etc.
with color differences in centers and margins
6. Surface: Glistening, dull, mucoid, etc.
7. Density: Opaque, translucent, transparent, other
8. Consistency: Butyrous, viscid, membranous, brittle, etc.

Reactions in the media include -

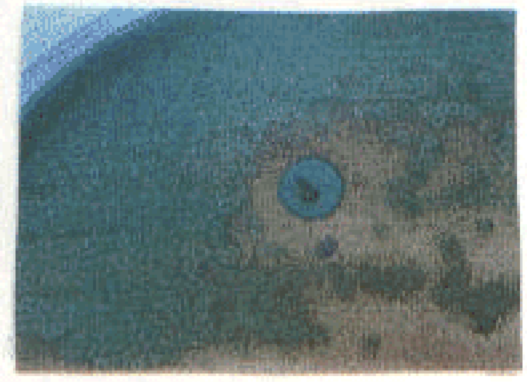
1. Hemolysis of blood	Alpha - partial clearing of blood around colonies with green discoloration of the medium
	Beta - Zone of complete clearing of blood due to lysis of the red blood cells
	Gamma - No change or lysis
	Double zone - halo of complete lysis with a second zone farther out of partial hemolysis
2. Pigment production	Water soluble pigments
	Fluorochrome pigments
	Pyocyanin
	Non diffusable pigments confined to the colonies
3. Reactions in egg yolk agar	Lecithinase - Zone of precipitate in medium surrounding colony
	Lipase - Pearly layer around colony visible by reflected light
	Proteolysis - Clear zone surrounding colonies

4. Added dyes, pH indicators, and aids in differentiating enzymatic activities.

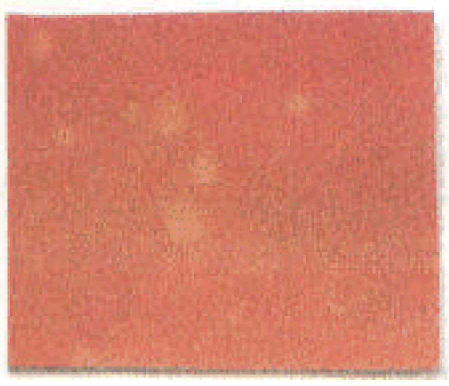
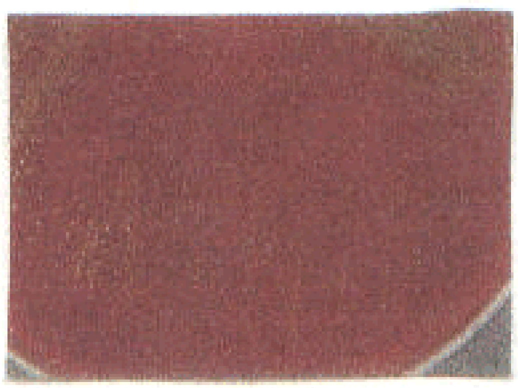
Examples of agar growth and descriptions of the colonies and species -

Convex, entire edge, 2 to 3 mm., creamy, yellowish, zone of beta hemolysis — Staphylococcus



Umbilicate or flat, translucent, butyrous or mucoid, broad zone of alpha hemolysis — Pneumococcus

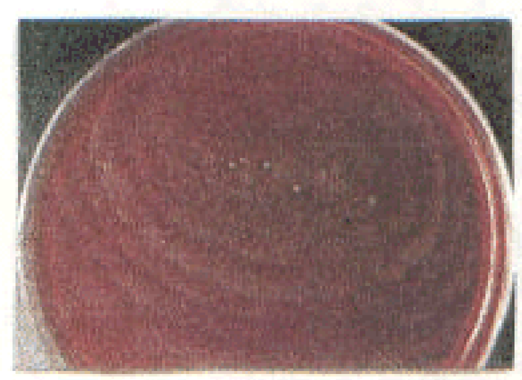
Convex or pulvinate, translucent, pinpoint in size, butyrous, wide zone of beta hemolysis — Streptococcus



Flat, opaque, gray to greenish, margins erose or spreading, green-blue pigment, grapelike odor — Pseudomonas



Pulvinate, semi-opaque, gray, moist to somewhat dry. Beta hemolysis may or may not be present. — *Escherichia coli* and Enterobacteriaceae



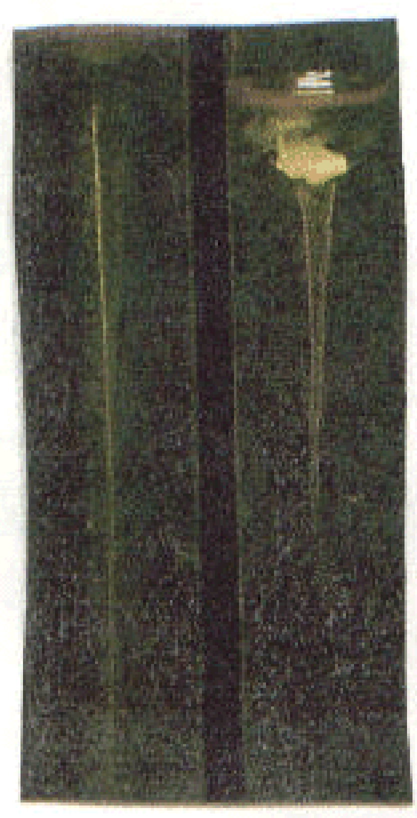
Flat, gray; spreading as thin film over agar surface; burned chocolate odor — Proteus

Nutrient agar growing gray-white, opaque colonies with the comma shaped (medusa head)



Left-Gelatin dissolves in hot water and gels when refrigerated. If it is "hydrolysed" it does not gel but remains liquid when refrigerated. The B.anthracis in the test tube on the left did not hydrolyse the gel while the B. Cereus on the right did. Right-If the organism will grow at 22 C you can stab it into the gelatin. B anthracis on left grows as a series of spikes "the fir tree", while the B. cereus has produced a funnel shaped liquifaction near the top.





Anthrax (Bacillus Anthracis)

History and Recovery from Nature

Anthrax is an aerobic, facultative anaerobe, gram positive , non-motile, spore forming bacillus rod. It has existed and is known to cause disease in animals and man since written records have been kept. It is believed to be one of the seven plagues suffered by the Egyptians in the time of Moses and was clearly described by the ancient Greeks. It is distributed widely worldwide and apparently arrived into the North American continent with livestock brought over from Europe. Humans are infected accidentally through contact with contaminated animal products. It is spread solely from the nose, mouth, anus, and tissues of infected and dead animals or humans. It forms spores that can last for decades and are highly resistant to heat, pH changes, and humidity and this accounts for the near impossibility of eradicating it from geographic areas. The anthrax spores have been isolated from naturally infected soil 60 years after samples were taken from the ground.

In the 1850's, the bacillus had been observed in the blood and organs of dying animals and by 1857 it was first successfully transmitted by the inoculation of blood from infected animals into new hosts. The Anthrax bacillus was first isolated in 1877 from the blood of infected animals and was cultured by Koch on the aqueous humor of an ox's eye. He described its life history, grew it in pure culture and used it to infect other animals to confirm its ability to reproduce the disease. Its toxin was not isolated until the 1950's when it was finally identified and understood. This led to a protective vaccine for humans. In 1881, Pasteur weakened the anthrax bacilli sufficiently by growing them at elevated temperatures (42-43 C) and this allowed for the development of an effective vaccine for animals.

Man is infected with anthrax in nature by dermal, respiratory, and ingestion routes. It is almost always transmitted from infected animals to human beings. From 1945-1970 there were 569 cases of anthrax in the United States. This number dropped to only 15 for 1970-1976. This was due mainly to effective vaccination programs of animals in high exposure and high risk areas and the effective treatment and disinfection of animal products from which the spores would found.

Hides, shaving brushes, textiles, and so on were imported into the U.S. early this century from South America and Asia without being adequately treated and this resulted in many cases of infected textile workers and consumers. Horses even contracted anthrax from infected wool on the underside of saddles. The bacilli were destroyed by soaking the brushes in formalin at 110 F for 4 hours and the textile mills were fumigated with vaporized formaldehyde. Solutions of 2% Formaldehyde are commonly used as disinfectant today. Anthrax has even been isolated from the hide covering bongo drums from Africa.

Pure cultures of the anthrax also occasionally infected laboratory workers. The death rate from the untreated anthrax in dermal infections averaged about 20%. Infection by the other routes resulted in much higher mortality rates reaching 90% in pulmonary cases and 100 % in intestinal cases.

In England in the mid 1800's, the wool and fleeces of sheep imported from the Far East contained many spores which often floated in the air resulting in many cases of pulmonary anthrax and became known as "wool sorters" disease. It first appeared as a pneumonia and often passed into fatal septicemia and meningitis. The inhaled spores were carried to the mediastinal lymph nodes where they germinated and began to infect and spread to other tissues, most notably the spleen.

Anthrax was found in the nose and throat of healthy persons exposed by inhalation in these industries without producing infection and serological testing indicated that a subclinical anthrax infection had taken place resulting in increased resistance among these individuals.

The anthrax spores are found in infected soil and vegetation and are ingested or inhaled by mostly domesticated herbivores. Occasionally, they gain entrance through abrasions in the hides and germinate at the site. Death usually occurs within three days of this germination. When infected humans survive, they are permanently immune except for one particular strain found in areas of Africa that has been found to be weakly immunogenic and resulted in repeated attacks of the same individuals.

Infected animals that died and were rendered often had spores survive the cooking, ending up in the meat and bone meal, and were then ingested in animal feed. This then resulted in further infected animals and replenished the reservoir of spores in the envoirenment. The dead animals identified with the disease are now buried with quicklime or cremated to stop spore distribution.

Anthrax produces a toxin that requires three factors to be virulent. It is the response to these three factors that induces the production of protective antibodies and it was the complexity of these three factors that made it difficult to develop an effective human vaccine.

Anthrax spores are found in most countries on earth. The soil is the principal reservoir with the disease in animals increasing the concentrations during outbreaks. Anthrax usually cycles in areas where heavy rainfall is followed by severe drought or vice versa. An animal-soil-animal cycle becomes established in dry areas where the spores are found in alkali soils. It is now known that the spores can survive indefinitely in dry environments in various dusts, on strings and swabs in laboratories, and blood spots on clothing. Once a suitable soil is infected with anthrax spores, it is known that the spores persist and can infect other animals.

Anthrax occurs and persists in soils where the pH is higher than 6 and the ambient temperature is above 15.5 C. Conditions of heavy rainfall create incubator conditions where water stands long enough to kill off standing grass. Dried up ephemeral watercourses or hillside seep areas that are calcareous (calcium or dolomite) or alkaline accumulate organic material during the heavy rains. Without these incubator areas, anthrax does not persist and infect livestock.

The combination of temperature, moisture, and pH will cause the spores to germinate and vegetate when these conditions are favorable. Acid soils and biological competition eliminate the anthrax in other envoirenments. Incubator areas do not form when there is intermittent rainfall during the warm months or during prolonged dry periods.

Environments studied that supported anthrax growth during outbreaks include -

Craig County, Oklahoma which is mostly rolling prairie with narrow wooded stream bottoms and broken escarpments. The soils in these areas that dried up following heavy rainfalls were the incubator areas that developed from or were influenced by the presence of limestone. It was found to be absent from adjacent sandstone and shale that ran across the county from southwest to northeast. In the 1957 outbreak, the area had suffered 3 years of drought lowering the groundwater and leaving only a few small ponds. Grass grew in all but the deepest portions of these ponds. In 1957, a prolonged, wet spring kept these ponds full creating ideal incubator conditions.

In the valleys, grassy washout areas and ponds behind levees also created some incubator areas. The outbreak began on high ground and spread to lower ground with the spores apparently dispersed by the flood waters. The anthrax outbreaks did not occur on haylands and well drained fields or where shale and sandstone occurred. The drying up of ephemeral streams after the wet spring resulted in new epidemics near Pryor, Oklahoma and Mount Valley, Kansas.

The Sedalia cattle trail was the route that cattle drives took out of the anthrax areas of Texas and Louisiana. The eastern fork of this trail passed over acid soils and did not support anthrax spores. The western fork from Pryor, Oklahoma to Baxter Springs, Kansas was heavily traveled until it was closed in 1871 because of the completion of the railroad. Eighty six years later, under ideal conditions, the spores infected animals in these new outbreaks.

Wayne County, Illinois suffered an outbreak in 1959 and it was determined that anthrax was endemic in portions of this county. Calcareous hickory soils were present on most farms which recorded cattle losses due to anthrax. Alkaline slick spots on grey silt loam over tight clay were involved in losses on other farms. The areas that dried out last were the last to experience outbreaks. In other words, the higher ground dried first with resulting incubator areas followed by the lower ground. The glacial till plain of the county dissected by Elm Creek was found to have the greatest anthrax potential. The hickory soils found in contours in the area did not hold water well enough to be cultivated so they were used as pasture and timber and formed incubators for the spores.

Gray silt loams with poor subdrainage and Bluford soils with good drainage saw the soil profile in these areas flooded, and created good incubator areas due to the prolonged surface runoff. The endemic areas with reservoirs of anthrax can be found between the flat prairie upland and steep gullied land along streams.

Other locations in the United States where anthrax may be found in the soils would be downstream areas of the many tanneries and animal hide textile areas that disposed of wastes in the local rivers and streams during the early parts of this century.

Glaciated calcareous soils and alkaline groundwater can provide incubators in anyplace that cattle have grazed. Pastures in the northeastern US, calcareous soils of southern Florida that also provides rockland environments, northern valley and ridge portions of the Appalachian Plateau with alkaline pH all provide reserves of spores.

The rich alluvial soils of the lower Mississippi river valley have often become incubators when dry conditions follow flooding. Epidemics in 1954 below New Orleans, and in 1958 east of the Ouchita river in Louisiana resulted in large cattle losses. In 1961, many outbreaks occurred on high ground northwest of Pearl River along the Natchez Trace of Mississippi. All of these were associated with calcareous soil areas.

Soils in Texas and Louisiana that are rich in both minerals and organic material and that have high pH particularly in the coastal regions also provide incubators that allow anthrax to perpetuate itself in these soils. The alkaline rivers of the southern great plains produce local accumulations of alkaline salts in many areas.

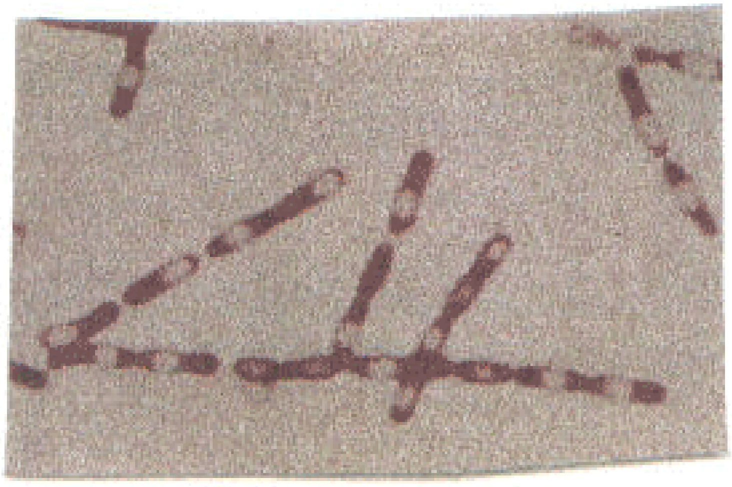
Anthrax is endemic on the dendritic drainage of the Niobrara and White rivers and the playa areas and valleys of small streams near the Missouri River in Nebraska and South Dakota. It occurs in all areas of the midwest that were concentration points for the big cattle drives of the 1800's and have the alkaline soils and organic material to serve as incubators. It occurs on the east and west Charlie Creek in eastern Montana where outbreaks in 1957 were associated with incubator areas created along the mature stream valley terrains.

The Green River Drift is a trail that connects summer and winter cattle regions near Pinedale, Wyoming that was the site of outbreaks in 1956. Abandoned beaver ponds created rich organic soil deposits needed for the anthrax incubator areas. The rapid heating of the soil (drought) heats the soil sufficiently to support germination (15.5 C ambient temp.)

Epidemiological studies of the soils of California and Nevada have concluded that anthrax will never be completely eliminated in this area of the United States. The meadows area of Fallon, Nevada which was a rest area on the Humboldt trail has considerable anthrax in its alkaline soils as does almost all areas of rich organic material, alkaline conditions, and a history of cattle populations. In 1968, 165 cases of anthrax were diagnosed on 34 farms in California.

US Government weekly "Morbidity and Mortality Reports" provides good records for the incidence of anthrax outbreaks throughout the country and are available at most Universities in the United States. In addition, virtually all state Universities have excellent records detailing soil types and cattle history throughout each area which are invaluable in evaluating anthrax recovery.

Gram stains of B. anthracis containing endospores. The one on the left is similar in appearance to clostridium species. Notice the squared off appearance of the individual cells and the formation of chains.



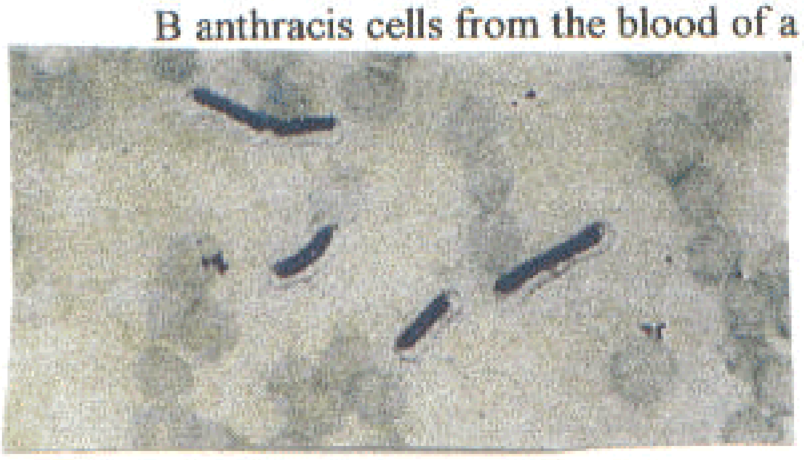


Vegetative cells on the left stained red and spores in the right picture stained green.



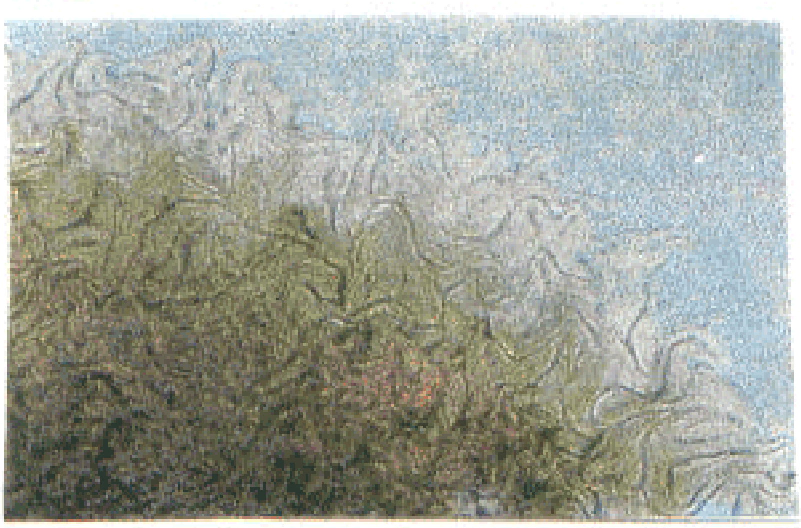


B anthracis cells from the blood of a guinea pig on the left. Colonies on blood agar-right.





The edge of an anthracis colony under low power of the microscope with the curled hair appearance. Right- filamentous or knotted string look when poured into the culture during preparation and observed at 18 hours.





Toxicity and Harm

Anthrax bacilli produce a toxin consisting of three identified factors. All three factors must be present in order for the toxin to cause maximum harm. These are -

1. Protective Antigen (PA)
2. Lethal Factor (LF)
3. Edema Factor (EF)

All three of these components are serologically active and distinct and immunogenic. A combination of PA and LF are required for lethal infections, PA and EF cause infection (edema reaction), while EF and LF have no effect. The mode of action is that PA reacts with receptors on the host cell surface to produce changes that permit EF and LF to enter the cell. EF then reacts with a heat stable substance which leads to the edema response.

The toxin alone is not sufficient for virulence. It also depends on capsule formation which the bacteria produces in the infected animal. It does not produce them on ordinary media. It has been learned that when sodium bicarbonate is added to the media (alkali) and 5% CO2 gas is added to the atmosphere, capsule formation can be artificially induced and this is what is used to make lethal cultures of anthrax for military use. Colonies grown under these conditions appear mucoid in texture. Spores can be formed in these special cultures, in the soil, and in the tissues and exudates of dead animals but are not produced in the blood and tissue's of living animals.

The vaccine developed by Pasteur involved repeated heating and culturing at 42.5 C. This damaged temperature sensitive plasmids of the toxin producing areas of the anthrax resulting in its inability to produce toxin. Killed vaccines have not been effective in humans and only attenuated or virulent strains used in inoculations and combined with protective anti serum have been found effective. Vaccination with the purified protective protein antigen is considered much more effective than attenuated vaccines.

Only strains of B. anthracis that produce both a capsule (spore) and a toxin are considered virulent. When anthrax infects a host, antibodies are produced against the capsular antigen but are not protective against the toxin. The toxin gradually accumulates until it causes death in the host animal from respiratory failure and anoxia by its action on the central nervous system.

On ordinary culture media, the anthrax grows but does not produce its exotoxin. The anthrax bacteria itself is not toxic either. That is why vaccines prepared with killed anthrax cells were ineffective at providing immunity. The three factors identified above require live animal tissue to produce the toxin. In nature when the spores germinate and vegetate in incubator areas, they do not produce these factors or toxin.

When the organism gains access to tissue through a cut or abrasion, they multiply locally with a fairly dramatic inflammatory response. Two to five days after infection, a small papule develops that quickly becomes a vesicle filled with dark bluish, black fluid. Rupture of the vesicle reveals a black eschar at the base with a prominent ring of reaction around the eschar. This is called the malignant pustule. If the carbuncle is incised and drained or surgically removed, mans natural resistance will usually prevent further spread of the infection.

When inhaled, they multiply in the lung and are carried to the draining hilar lymph nodes where marked hemorrhagic necrosis may occur. They are also carried by the bloodstream to the spleen where they multiply in enormous numbers. In addition, alveolar phagocytes attack and ingest the spores and carry them to the mediastinal lymph nodes where they not only have survived the phagocyte attack but now germinate. From this site, the cells spread throughout the body. The symptoms are those of the typical respiratory infection with fever, malaise, myalgia, and unproductive cough. Within several days it becomes serious with marked respiratory distress, septicemia, meningitis, and cyanosis. Death usually occurs within 24 hours of this worsening. Only a small number of spores are required to be infective by the inhalation route.

Ingestion of spores that survive the stomach acids result in invasion of the gastrointestinal mucosa with ulcer formation. Nausea, vomiting, and diarrhea with occasional loss of blood is observed. This is followed by profound prostration, shock and death

From all three locations, invasion of the bloodstream may occur with profound toxemia and fatal meningitis. Septicemia and toxic effect on the central nervous system results from the release of toxin and organism into the bodies tissues.

Anthrax infections in humans who survive provides complete immunity in all individuals except for the African strain cited earlier. The blood serum of surviving animals and humans carries protective antibody to high titer.

In lower animals, the most sensitive are the rabbit, guinea pigs, and white mice which are infected with lethal doses with only a few bacilli or even a single bacilli in the mice. Rats possess a marked resistance to anthrax while the carnivorous animals and humans show some resistance. Feeding experiments in animals using the vegetative form of anthrax without the spores results in no infections because the non spore bacilli are killed by the stomach acids. The spores survive the acids and produce lethal infections at larger doses.

Anthrax is about 80% fatal in cattle and sheep with the bacteria multiplying enormously in the blood and internal organs. The spleen becomes enlarged and takes on a deep red color. In the more resistant animals, the anthrax infection usually remains localized and does not spread. This resistance is of two types. The prevention of spread is an antibacterial resistance, while the resistance to the toxin inhibits the injury it can cause to the tissues.

Toxin can be produced for use as a weapon in large scale by

A) Infecting guinea pigs with spores and collecting the thoracic and peritoneal exudates immediately at death. These are mixed, centrifuged to remove guinea pig and bacterial cells, and sterilized by filtration before purification.

The toxin is then precipitated from the plasma-exudate with barium acetate and ethanol. It is solubulized and again reprecipitated with ammonium sulfate to obtain a 50 fold increase in purification. Recovery yield is about 20%. Total yield is about 2 mg. of purified toxin from 20-30 large guinea pigs of app. 84% purity with the balance being guinea pig plasma components.

B) Producing toxin directly from the bacteria on culture plates using sodium bicarbonate in place of serum albumin, blood aerated with 20% carbon dioxide, charcoal, gelatin in place of serum, or a simple salt medium with proline and threonine for protective antigen production. It is known that bicarbonate is required early in the growth cycle and that it affects cell permeability and allows release of the toxin from the cell into the medium. Without bicarbonate, toxin production is completely inhibited from release through the cell wall although production can take place with the bacillus retaining it internally in the cells. CO2 is required for toxin production to take place at all. This is why the high CO2 content of human and animal tissues permits the production of toxin and makes it deadly.

It is known that virulence is increased 200 fold when sterile plasma exudate collected from guinea pigs dying of anthrax was injected into animals with spores. This may be useful in combining both toxin and spores in dust based weapons. Even attenuated anthrax can be made deadly by the inclusion of even a small amount of toxin accompanying the live cells. The toxin itself evidently interferes with the serum phagocytic and bactericidal activity. The toxin not only kills but also helps the anthrax bacteria overcome the host defenses and this makes them much more effective at colonizing tissues.

The application of toxin affects how quickly it can kill. 10,000 rat units of toxin injected by IV into monkeys caused death in 28-54 hours. Injecting 1,000 units directly into the cerebral spinal fluid produced death in 6-10 minutes. Tetanic paralysis and respiratory failure occurred immediately on injection.

One of the unique aspects of anthrax is that the blood oxygen levels fall to 1% from normal levels of 18-21% for arterial blood and 12-15% for venous blood. In all other diseases including death by drowning or asphyxiation, the oxygen level at death remains above 5%.

Host	Spores to infect	Lethal dose units/toxin	Time to death (hours)
Mouse	5	1,000	24
Guinea Pig	50	1,125	24
Rabbit	5,000	2,500	72
Rhesus Monkey	3,000	2,500	28
Chimpanzee	unpublished	4,000	60
Rat	1,500,000	280	20
Dog-Beagle	50,000,000	60	20

Protection, Immunity, and Resistance

Vaccines prepared with killed bacilli do not produce an effective immunity because it is not the organism that caused the harm but the presence of the three immunologically distinct toxic factors we have already described. None of these were toxic when tested alone. Only when their is toxin production in tissues is their an ability to produce effective antibodies. Only when attenuated, or virulent/partially virulent strains have been used in inoculation, in conjunction with protective anti-serum, has effective immunity been achieved in animals. A living spore vaccine made from a non encapsulated strain of anthrax has replaced Pasteur's original vaccine. The living vaccine gives good protection but causes some local cases of the disease. An alum precipitated protective antigen is used in conjunction with this vaccine for more effective protection in outbreak areas and is used in human vaccines as well.

Anthrax was treated with partial success in animals and people with a combination of arsenicals and anti-serum before the development of antibiotics. Sulfonamides have been effective and penicillin very effective in treating anthrax. Some strains of anthrax are penicillin resistant and tetracycline's, streptomycin, and cotrimoxazole are good alternatives. Skin lesions from anthrax must not be incised and drained as would be expected with Staph infections because this can lead to widespread dissemination of the anthrax to surrounding tissues and the envoirenment.

Penicillin V for cutaneous infections is given orally at 30 mg/kg of body weight daily in four equal doses for five to six days. Aqueous penicillin G-5 million units, is given intravenously every six hours with streptomycin at 500 mg intramuscularly every 12 hours for inhalation or gastrointestinal infections. The use of antibiotics has reduced the fatality rates of dermal anthrax from 20% to 5%.

The microscopic cells of Clostridium Tetani. Cells have terminal spores that are spherical when mature and much greater diameter than vegetating cells. This produces the classic drumstick appearance of this species.

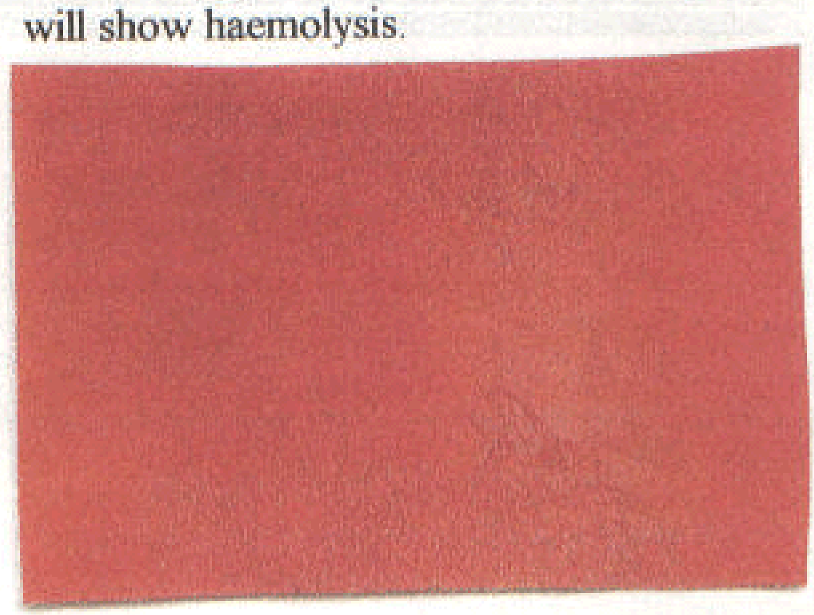




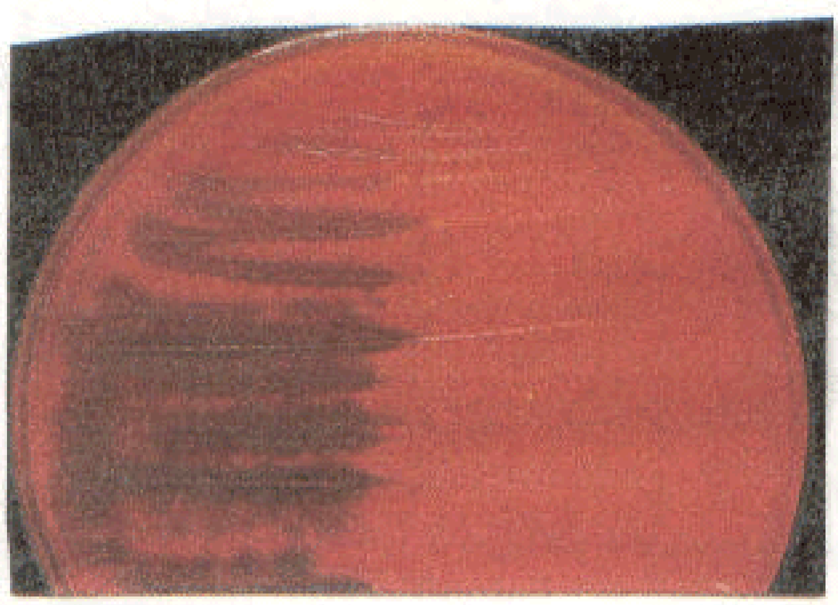
C. Tetani on stiff agar (4%). which retards the swarming so it can be isolated from other bacteria in mixed cultures [Using extra gelatin at 2-4 times the label directions has a similar effect.].



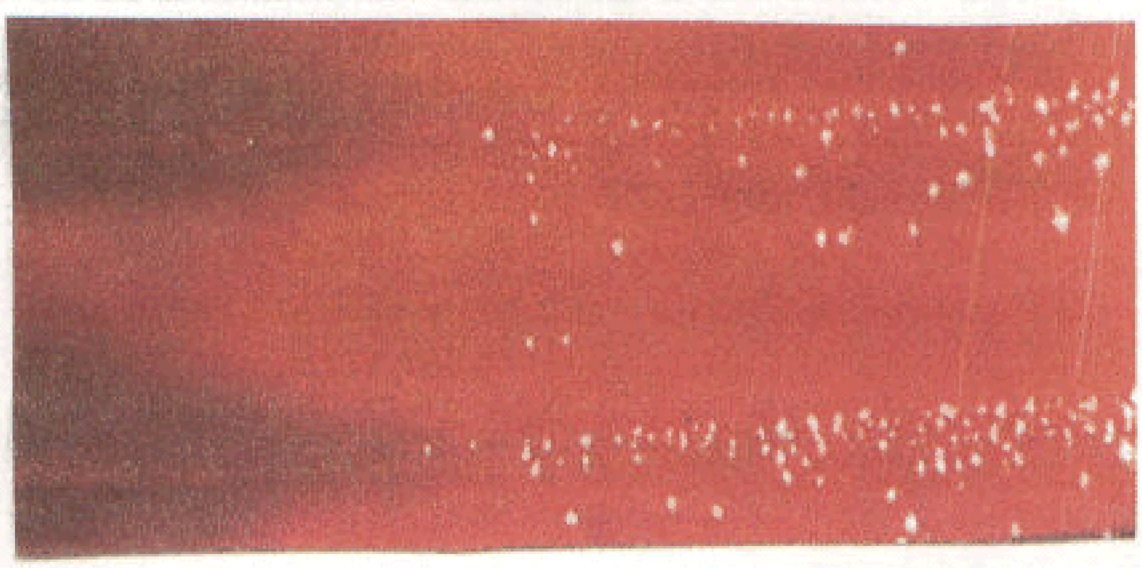
Spreading flat colonies barely discernable with projecting filaments swarming across the blood agars surface. Later, dense growth will show haemolysis.



C. Tetani growing across a blood agar plate treated with antiserum on the left half. Although growing throughout the plate, the antitoxin inhibits the haemolysis of the blood cells



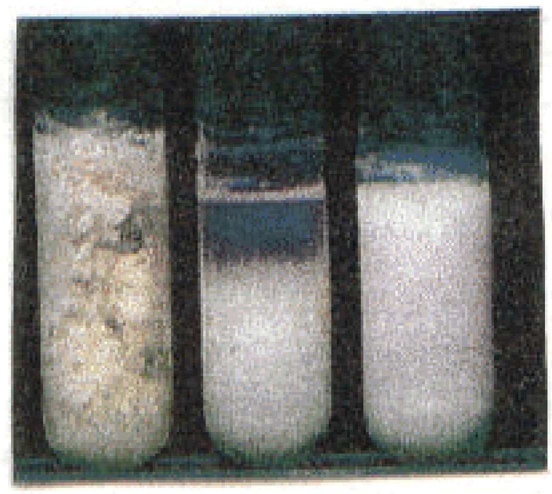
Close up of the border between the two zones which shows a change in colony morphology. The antiserum also contained antibody which inhibited the swarming spread leaving isolated colonies.



C. welchii is not motile and does not spread through the medium in the gel stab below while the C. bifermentans on the right has.



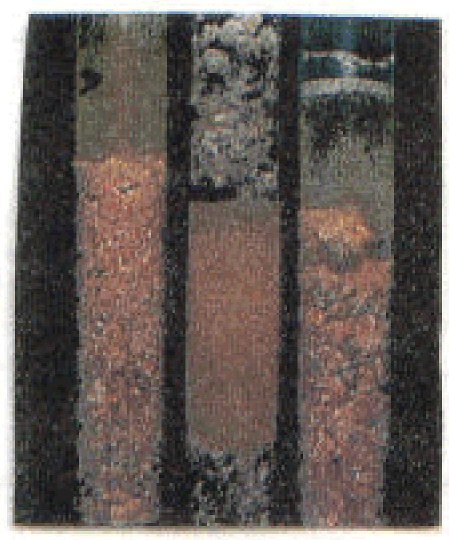
The "stormy clot" of the C. welchii on left in litmus milk. The medium is disrupted by the gas production.



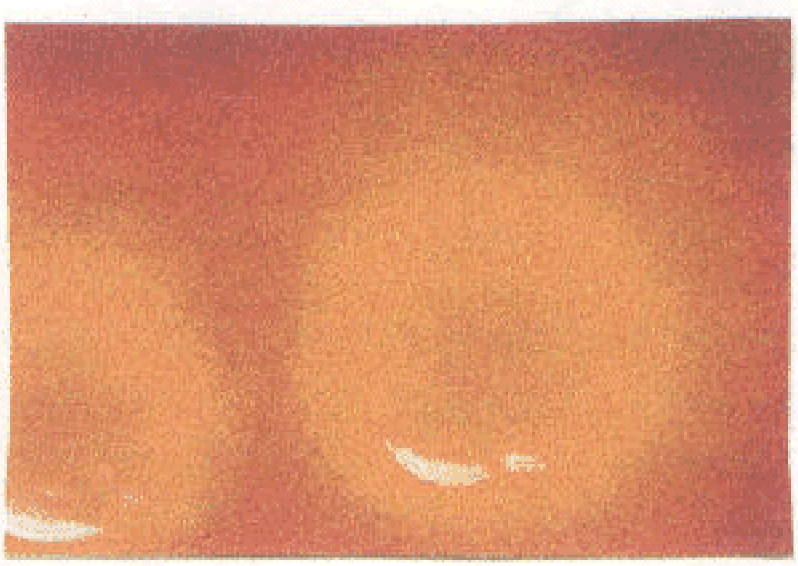
C. welchii on the left in molten glucose agar solidified and incubated. All three species produced gas with welchii the greatest followed by the tetani on the right and the C. sporogenes in the middle.



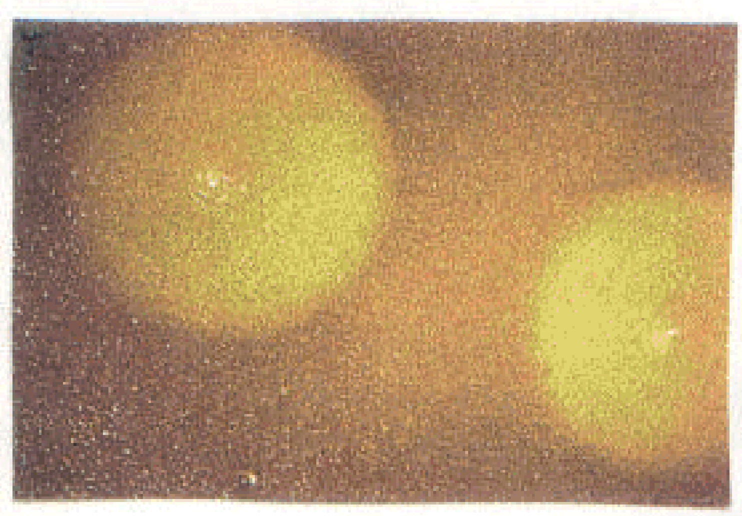
Cooked meat medium with C. welchi on the left turning the meat pink-red, the C. sporogenes turning it black with digestion of the edges and a putrid odor. C. tetani on the right. All three produced gas.



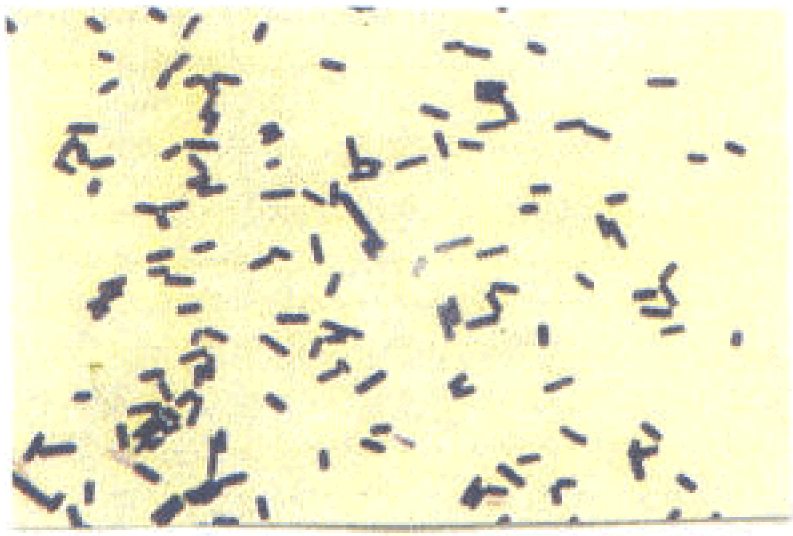
Colony of C. welchii grown on blood agar showing both zones of hemolysis.



C. welchii on egg yolk agar with zone of precipitate.



Gram smear of C. welchii with single cells which are stout, short, and have truncated ends with no spores.



A 24 hour colony on blood agar. No spores and some older cells stain red.



Stain of cells from 24 hour thioglycollate broth culture. No spores and a few filamentous forms.



C. welchii on blood agar after 24 hours at 35 C. There is a double zone of hemolysis with the inner, complete zone due to theta toxin.

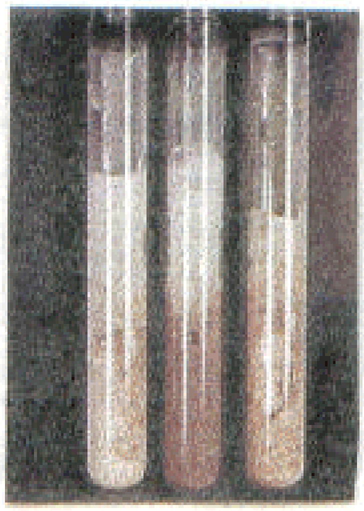


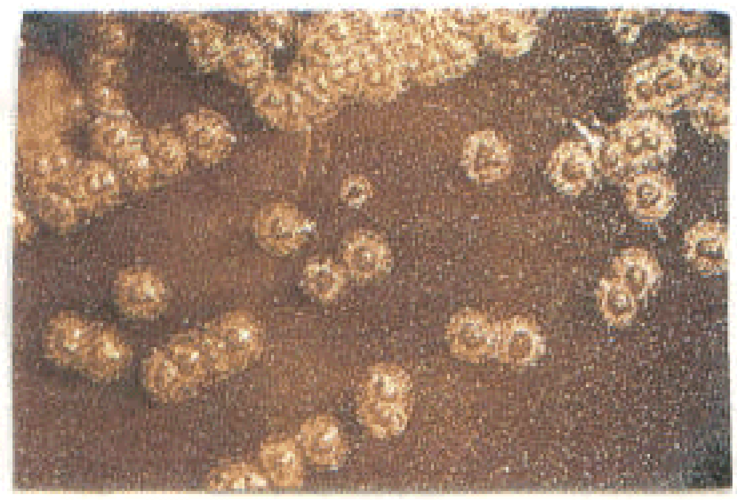
Strains have been mutated to spore on ordinary media by treating the media with nitrosoguanidine (the explosive described in Volume 3).

Some strains grow best at 45 C with regeneration times as low as 10 minutes. C. welchii will survive low exposure to oxygen and even grow on low oxygen tension blood agar. It will usually outgrow all other organisms at 45 C in chopped meat medium during the first 4-6 hours of incubation and its colonies are visible at this time. This makes it one of the fastest growing bacteria. Blood plates streaked after this time will have proportionally larger numbers of C.

Left: The test tube in the center illustrates coagulation and digestion of the milk by a proteolytic species of Clostridium.Coagulation and gas production occur on both sides which is usually called "stormy fermentation"

Right: C. Botulinum Type A exhibiting the irridescent "pearly layer" on the surface of the colonies extending into the medium surrounding them. This is the lipase activity on egg yolk agar.





The surface colonies on agar are large, grayish, irregular, semi-transparent, with a central nucleus and a reticular or fimbriate border measuring 5-10 mm in diameter. The edges are translucent, with glistening colonies that are often seen with a thicker brownish center. It is hemolytic and filamentous on blood agar. The semi transparency of the colonies helps differentiate them from opaque colonies of other sporing organisms. Some toxic strains have been observed to mutate to non-toxic sporing colonies.

Gelatin is liquefied by Clostridium, while agar stabs produce a white line of growth which stops short of the surface and has short lateral spikes or radiations. The gas production is considerable and evident by the parts of the agar pushed apart, especially when glucose is added to the agar. It can also be grown on artificial mediums with several amino acids added. When brain, meat, or coagulated proteins are added, they turn black as they are digested by the Clostridium botulinum.

Coagulated serum is slowly liquefied, milk casein is digested, milk is peptonized, and meat is digested and usually blackened by toxins A, B, and F strains. Types C, D, and E, are generally non-proteolytic. It will ferment glucose and maltose and Type A frequently ferments salicin and glycerol. Type B ferments glycerol but not salicin, and Type C does not ferment either. All types produce lecithinase enzyme. On blood agar all strains are beta hemolytic except type G.

Type A strains and most type B in the United States are usually proteolytic. The European strains of type B are usually non-proteolytic.

Clostridium Species

Members of the Clostridium genus are widespread in nature and cause disease in man. Some of the deadliest toxins known are produced by members of this genus. These include -

Organisms causing -	% Single species recovered	% with more than 1 recovered
Gas gangrene		
Clostridium welchii [perfringens]	(30%)	60%
Clostridium oedematiens	(5-17%)	20-40%
Clostridium bifermentans [sordellii]	less than 1%	
Clostridium histolyticum	" " "	
Clostridium fallax	" " "	
Clostridium Septicum		10-20%

Clostridium Tetani - the cause of tetanus
Clostridium Botulinum - the cause of food poisoning (botulism)

With only a few exceptions, all the bacteria producing powerful and deadly exotoxins belong to this genus. All the strains produce spores and can be preserved by freeze drying or storing in cooked meat broth containing chalk and minced cooked egg white.

All these organisms stain gram positive but gram negative forms are seen in older cultures. They are all motile with peritrichous flagella except Cl. welchii and must be observed under anaerobic conditions. Pasteur first isolated and studied Clostridium (butyricum) and the by product butyric acid in 1861. Little was known of the spore formers until the large number of gas gangrene infections from bullet wounds prompted large scale research resulting in the discovery of many new clostridia.

The members of the genus can often be told apart by the conditions in which they form spores. C. perfringens seldom form spores unless grown on media containing magnesium sulfate which results in oval subterminal spores. C. tetani readily produce spores that extend two to three times the width of the bacterium itself which are terminally located and give the tetani its characteristic drumstick appearance. C. botulinum sporulates more readily at 25 C than at 37 C. C. perfringens is the only non-motile member of the genus and with C. septicum are encapsulated when grown in media containing serum and serous exudates.

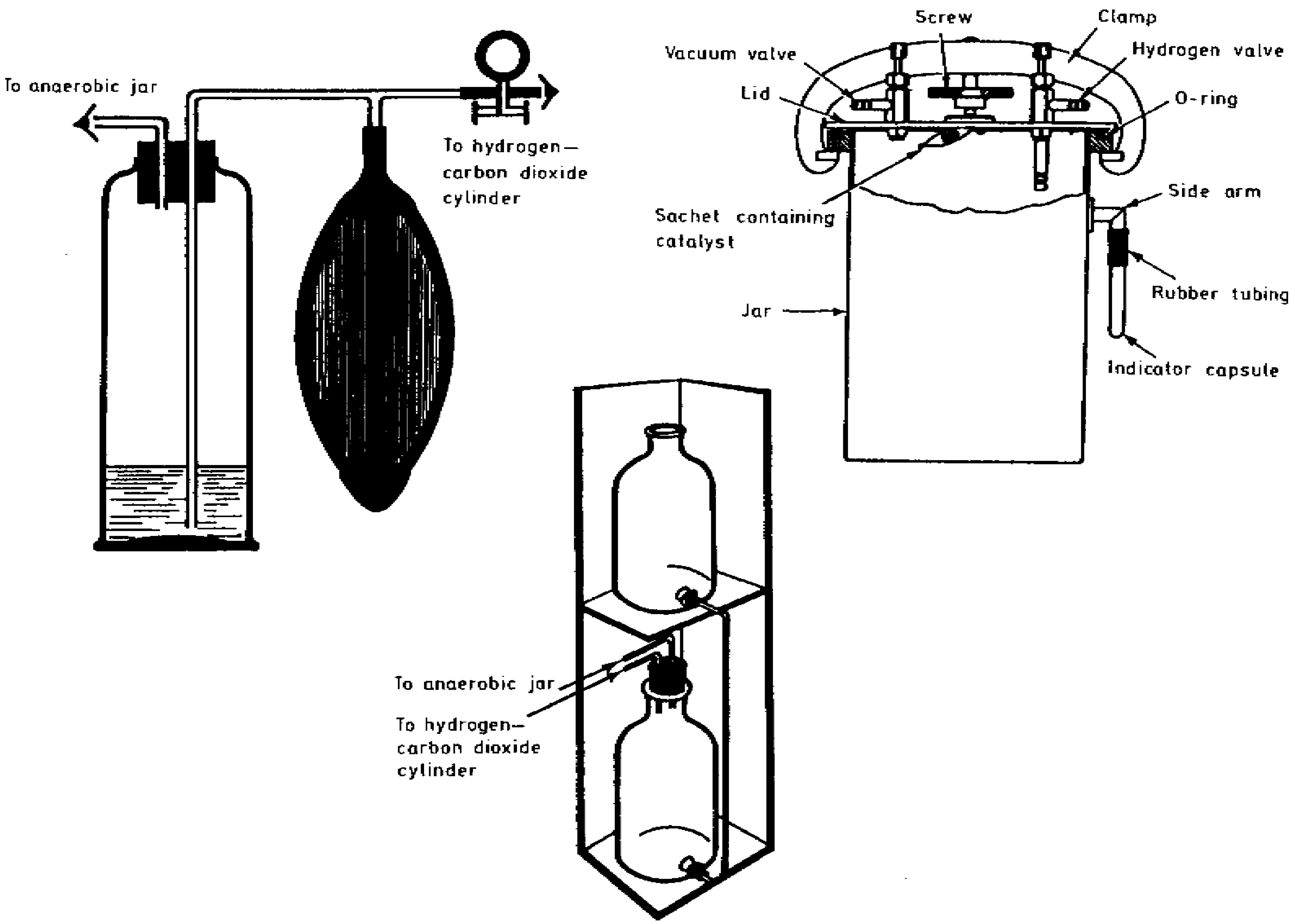
All Clostridium grow on blood agar, peptone water or meat infusions. The media must be freshly prepared since they will absorb oxygen from the air and become toxic to the anaerobes. Liquid media can be boiled off prior to use to drive off absorbed oxygen. C. perfringens and histolyticum are microaerophillic while all other species are strict anaerobes. On solid media most strains grow slowly sometimes only producing a thin, effuse, slowly spreading film on primary isolation.

For most species, the optimal pH is 7-7.4. Some species are thermophillic preferring temperatures of 50-60 C.

	Morphology in culture*	Colonies on Blood-agar	Cooked meat medium	Milk medium	Lique-faction of coagulated serum	Fermentation of					Pathogenicity to guinea-pigs and mice
						Glucose	Lactose	Sucrose	Maltose	Salicin	
Cl. tetani	Slender bacilli with round terminal spores	Transparent, with long feathery spreading projections; usually haemolytic	*Slight* digestion, blackening and pungent odour	Unaltered	− (but may be softened)	−	−	−	−	−	+ (tetanus produced)
Cl. tetanomorphum	Resembles *Cl. tetani*; round terminal spores	Small and transparent, with irregular outline	Gas; no digestion; no putrefactive odour	Unaltered	− (but may be softened)	+	−	−	+	−	−
Cl. welchii	Large, thick, often rectangular bacilli; spores usually absent	Large, circular, with regular outline; haemolytic	Gas, no digestion, meat reddened	Acid, gas, rapid clotting; "stormy-clot"	−	+	+	+	+	(−/+)	+
Cl. septicum	Large bacilli with central or subterminal spores†	Transparent, irregular, with spreading projections; usually haemolytic	Gas, no digestion, meat reddened	Acid, gas, slow clotting	−	+	+	−	+	+	+
Cl. oedematiens	Like *Cl. welchii* but somewhat larger and more pleomorphic; central or subterminal spores (not numerous)	Transparent, flat, tend to fuse and form spreading film; usually haemolytic	Gas, no digestion, meat reddened	Sometimes slow clotting	−	+	−	−	+	−	+
Cl. histolyticum	Filamentous in old cultures or if grown aerobically; spores large, oval and subterminal	Irregular, round, opaque, greyish-white colonies; haemolytic	Digestion of meat with evolution of H_2S; white crystals deposited late	Acid, gas; casein precipitated and digested	+	−	−	−	−	−	+
Cl. bifermentans'	Usually numerous sporing forms, often in chains; spores large, oval, central or subterminal	Round, crenated, irregular; usually haemolytic	Gas, digestion, blackening and putrefactive odour whitish mucoid deposit	Acid, gas, digestion	+	+	−	−	+	(+/−)	(−/+)
Cl. botulinum	Large pleomorphic bacilli; oval spores, usually subterminal	Large irregular colonies with opaque raised centre; usually haemolytic	Gas; types vary in proteolytic activity (*see* text)	Casein precipitated and digested by some types; others produce no digestion	(+/−)	+	(−/+)	(−/+)	+	(−/+)	+
Cl. sporogenes	Somewhat slender bacilli; central or subterminal spores	Usually irregular colonies with feathery projections; haemolytic young colonies small, circular, opaque.	Gas, 'digestion, blackening and putrefactive odour	Acid, clot, digestion, later alkaline	+	+	−	−	+	(−/+)	−

In order to recover and grow Clostridium species you must be able to create an atmosphere absent or low in oxygen which is poisonous to the bacteria. There are several basic ways of doing this while still growing visible colonies.

1. You can set up a cabinet or "glove box" with valves and hookups to CO2, helium, nitrogen, hydrogen, neon, and other inert gases from compressed cylinders. The culture is placed into the device and the desired gas is flooded into the chamber forcing the air out. This is basically accomplished with the use of the commercial gas pak jar or set up lab equipment as shown.



Carbon Dioxide is the gas most required for best production of Clostridium spores and is used in 10% or greater amounts by many other anaerobes. Usually a cylinder of compressed CO2 gas is used but it is recommended to use a mix of 90% hydrogen and 10 % CO2, or a mix of 80% nitrogen, 10% CO2, and 10% hydrogen. In liquid or deep solid media the CO2 can be directly mixed into the water before adding by using soft drink mixing heads without the syrup.

Other methods of creating a high CO2 atmosphere include -

2. In an air sealed container with the culture, for each 100 ml of container, a mix of 1 gram of pyrogallol and 10 ml of 2.5% sodium carbonate are mixed together in a test tube. The reaction absorbs the oxygen from the atmosphere with production of some carbon monoxide. The ingredients are sometimes simply added to moist cotton wool and placed inside of a small incubator to accomplish the same effect on the atmosphere.

3. Gram + organisms use up oxygen in a sealed envoirenment and it has been used in separate culture plates to consume and change the atmosphere so that in a day or two, the anaerobes can begin to grow.

4. Steel wool can be soaked in a solution of .5% copper sulfate with .25% wetting agent and sulfuric acid added to a pH of 1.5 to 2. This creates an activated wool that reacts and becomes coated with metallic copper due to rapid oxidation which removes the oxygen from the air. The wool is placed into the sealed bag or container with the culture medium.

5. The air can be vacuumed out of the container producing a very low pressure atmosphere. Chemical reactions of baking soda placed on the container bottom with a small dish of an acid in solution such as weak sulfuric, hydrochloric, sulfamic, citric, etc. This allows the dish to be turned over reacting with the bicarb to produce CO2 gas.

6. Creating a culture medium in depth such as the broth or deep tube which is stabbed to inoculate the material.

Clostridium Botulinum

History and Recovery from Nature

The organism Clostridium botulinum belongs to the group of spore forming anaerobic Bacillus that cannot grow in air. Under the microscope the bacilli occurs singly or in pairs and appear with rounded ends about 4-6 x 9-12 *u*m in size. The spores are oval and slightly bulging. The bacilli are motile with peritrichous flagella and stain gram positive unless decomposed.

References to the food poisoning caused by Botulism is made 1,000 years ago during the reign of Emperor Leo VI who banned the making and eating of blood sausage because of the outbreaks. Governments first regulated the making of blood sausage in 1793 to prevent illness although the cause was unknown at the time.

Botulism was first observed and recorded medically in Germany in 1785. It was responsible for many outbreaks of food poisoning in sausage and was finally isolated in 1896 and described by Van Ermengen. He found the Clostridium microbe to be a producer of potent toxin. The disease is characterized by speaking difficulty, inability to swallow, and double vision beginning at 12-24 hours to four days after toxin ingestion. It is not an infectious disease and its harm is caused by a toxin it produces while growing on foodstuffs ingested by animals and humans.

The organism produces some of the worlds deadliest toxins known as botulism or food poisoning. The toxins are broken down into antigenically distinct groups designated A,B,C,D,E, F and G. Types A,B, and E have caused the most problem in man although the others are also capable of harming man. All are useful as weapons. The "A" toxin has been isolated as a pure crystalline protein and on a weight basis may be the most toxic substance in nature. The lethal dose (LD50) for humans is estimated at .000000033 mg although the effect is slow and it often takes many days for the action of the toxin to cause death.

Type	Species Affected	Location of Type
A	Man	United States, USSR
B	Man, horse	U.S., Northern Europe, USSR
C alpha	Birds, Turtles	Worldwide
C beta	Cattle, Sheep, Horses	Worldwide
D	Cattle, Sheep	Australia, South Africa
E	Man, Birds	Northern Europe, North America Japan, USSR
F	Man	Denmark, US
G	None	Argentina

C. Botulinum is highly resistant to alcohol and one culture separation method employed is to mix one part of sample with one part of 100% ethanol and incubate at 37 C for one hour with occasional shaking. This is then inoculated into cooked meat broth which is incubated at 80 C for 10 or 20 minutes (or both in separate samples). They are then anaerobically incubated for 3-5 days at 30 C for best toxin production. The toxin positive samples are then transferred to egg yolk agar and placed in anaerobic conditions quickly as the Clostridium are very sensitive to oxygen. The addition of a small amount of "Neosporin" from a pharmacy onto the culture medium also helps stop aerobic organism growth in favor of Clostridia.

One of the interesting ways that C. botulinum is spread in nature is from the tissues of dead animals, especially cats, that have died and decomposed with their tissue residue diffusing into hay or silage feedstuffs that are then ingested by animals. The carcass toxin has been measured containing as many as 3,000 MLD (minimum lethal doses) per gram of tissue. It is also the leading cause of natural death of ducks in the western United States and this helps spread the organism from the dead carcasses to the surrounding environment.

Another source of spore production in nature occurs when strong winds tear aquatic plants from their moorings onto the shore leaving long rows of uprooted vegetation to decay. Aquatic invertebrates present in the decaying vegetation die from lack of oxygen and the C. Botulinum proliferates in their bodies. The ducks ingest part of the decaying vegetation and invertebrates which then kills them and the cycle goes on as fly larva pick up the toxin on their coat and internally which they spread to other species. Botulism in pheasants follows a similar pattern.

Growth and Production

Microscopically, it occurs singly, in pairs, and in chains. It is motile with 4-8 peritrichous flagella and its spores are subterminal and oval and often have a club shaped appearance.

Clostridium Botulinum grows on all ordinary media but when meat tissue from animals is added the growth is most rapid and abundant. They are strict anaerobes and can only be grown in the absence of air or oxygen. The easiest way to grow C. Botulinum is in cooked meat broth. It grows throughout the medium as long as the dissolved oxygen has been boiled out. It will not grow on or near the surface if the air above it contains oxygen. A butyrous scum is occasionally produced on the surface of the broth. Types A and G will digest the meat particles as well as some strains of B and F while the others do not attack the meat. Its ideal growth temperature is 20-40 C with 35 C being ideal. It usually doesn't produce spores at temperatures higher than 20-25 C.

On egg yolk agar, all types except G produce a restricted opalescence and pearly layer due to lipolysis. On milk agars, a zone of clearing develops due to proteolytic activity with types A, G, and some strains of B and F. All strains produce acetic and butyric acids as byproducts.

Most strains stand heat of 100 C for several hours and sometimes as long as 20 hours, but are destroyed at 120 C in a few minutes which is usually a method for pre separating Clostridium from Anthrax. The deadly exotoxin is produced in the culture media and in contaminated foods which causes the disease. The toxin is destroyed by heating at 80 C for 30-40 minutes or 100 C in 10 minutes. Each of the toxins (and strain of C. botulinum) are immunologically distinct so that the antitoxin must be specific.

The spores are also resistant to irradiation and can survive temperatures of -190 C. Initiation of growth with toxin production only occurs at a pH of 7-7.3. Toxin production is enhanced by the addition of casein hydrolysate or corn steep liquor to the medium.

Toxicity and Harm

The water soluble botulinum toxin is the most potent of all known exotoxins. Antibiotics have no effect on the toxin. It is a high molecular weight complex made up of a neurotoxin component combined with other non-toxic bacterial proteins. The nontoxic proteins and their respective weights are different for each type of toxin. These non-toxic portions of the complex act to protect the toxin from being destroyed by the stomach acids and the digestive enzymes (proteinases) after people consume it. All the neurotoxins of each type have similar pharmacological effects and structures but are immunologically distinct.

The toxin is not released by the organism during its life phases. It is produced and held internally in the cell until it dies and breaks down (autolysis). It is then released into the envoirenment. When it is consumed with foodstuffs, it is rapidly dissolved and passes from the small intestine into the lymphatic system before it is found in the bloodstream. Much of the ingested toxin however does not pass out of the intestine. Some of it is denatured and possibly destroyed by proteolytic enzymes while it is believed that many of the molecules are so large that they have difficulty physically passing through the membranes separating the tissues and getting into the circulation. There is in fact a great deal of difference in the ability of the different types of toxins to pass through the intestinal walls of the different host species. It is now believed that the toxin is absorbed most easily in the upper part of the small intestine and that it enters the circulatory system via the lymphatics.

The bacteria will produce toxin over three to four days and then level off. At this point, they stain gram negative rather than gram positive as they die off and lyse, releasing the toxin to the surrounding medium

The toxin passes through barriers and membranes in the body with difficulty and there is no penetration into erythrocytes. There is very little toxin found in cerebrospinal fluid at the time of death.

Another method employed is extracting the culture with 1% sodium acetate at a pH of 3.5, followed by fractionation with ethyl alcohol at 4 C. The final material is crystallized with 10-30% ammonium sulfate saturated in solution and the toxin precipitating in small needle like crystals.

Research also indicates that the amino acid tryptophan and its derivative 5-hydroxytryptamine (and possibly cysteine) have an antitoxic effect on the molecule when injected into mice.

Type B Toxin

Type B has only a slightly less toxic effect than Type A but is immunologically, chemically, and physically distinct form the A form. In 1957, a pure crystal of the toxin was obtained by precipitation with sulfuric acid, extraction with calcium chloride, and precipitation with ethyl alcohol at just above freezing temperatures. Precipitation with ammonium sulfate solution and separation on DEAE column cellulose at pH 5.6 also has since been used.

Type C Toxin

The only methods listed for the recovery of this toxin are the cellophane sac culture technique and a precipitation, dialysis, and salting out with 2 m NaCl at pH 2.0.

Using sodium chloride in solution to extract the toxin followed by adding metaphosphoric acid to precipitate it has also been employed. Another method is extraction with 40% ammonium sulfate solution precipitation followed by redissolving in distilled water and using calcium chloride to reprecipitate the toxin with 25% ethanol at -5 C for the final precipitation.

Type D Toxin

The cellophane sac culture technique has also been used on this toxin with successive precipitation and re-solution in ammonium sulfate solutions where they eventually obtained a uniform material. A single unconfirmed test reported a value of 5,000-20,000 times that of the toxicity of the A and B toxins and 8,000 times greater than a 90% pure D.

The culture and organism mix is dissolved in water and the solids filtered off. Ammonium Sulfate 40% is added to precipitate the toxin at pH 5.8. The slight precipitate contains most of the activity of the culture and is purified by repeating the procedure.

Type E Toxin

Protection by Antibiotics, Vaccines, and Immunity

The formol toxin described earlier can be used as an immunizing antigen to produce active immunity with circulating antitoxin in the blood. This has been used in some cases in animals of high value (such as race horses). Humans can be immunized with fluid or alum precipitated toxoid of type A or B. Naturally occurring botulism is so rare that active immunization is not practicable and so is not practiced Active immunity can be produced in man by giving mixed toxoid (A and B) in three doses at two month intervals. Similar immunization of animals has been carried out in Australia on a small scale.

The botulinum toxin crystals free of debris and dissolved in water can be treated with formalin solution of 1-1.5% for 5 days at 39.5 C to achieve an excellent antigen for antitoxin. For immunization, the toxoids are absorbed onto 7-8 mg/ml aluminum phosphate.

Antitoxin for prophylactic and therapeutic use in humans (10 ml given intramuscularly) is produced by animal immunization with toxoid and used to treat those suspected of ingesting poisoned foods. These usually contain antitoxin against types A, B, and E and is given twice at 10 weeks apart and a booster at 52 weeks. The toxoid is usually absorbed onto aluminum sulfate or mixed with Freunds adjuvant prior to treatment. In the food poisoning cases, this has a very marked prophylactic effect before the onset of symptoms but their therapeutic efficacy is marginal because, as mentioned earlier, the antitoxin has no effect after the toxin has translocated into the neural cells.

Clostridium Tetani

History and Recovery from Nature

Tetanus occurs in man and animals when a wound or abrasion becomes infected with the organism Clostridium Tetani. This organism produces a toxin that diffuses into the tissues resulting in exaggerated muscular responses, tetanic convulsions, arching of the back, extension of the extremities, and "lockjaw". Hippocrates observed the symptoms and described its course as "Death, if it occurs, follows relatively soon after the appearance of symptoms. Such persons as are seized with tetanus die within four days, or if they pass these, they recover". It was first medically described in 1884 and isolated in pure culture in 1889 where Kitasato described its relation to the disease. He also showed it had an inability to invade the blood stream. He also laid down the basis for antitoxic therapy when he discovered diphtheria and tetanus antitoxins which were also the first bacterial toxins to be discovered.

In undeveloped countries, neonatal tetanus is a serious problem at childbirth because of neglected umbilical cords and pregnant women should be immunized to protect the newborn. Otherwise, there is a significantly high mortality rate. The mortality rate is high even among vaccinated adult individuals who contract the disease.

Tetanus bacilli occurs in the intestine of humans and animals with those who work in and around the soil (farmers) usually containing spores in their feces, while urban populations typically do not. The use of human solid wastes as is practiced in China will increase the amounts present in the field. C. Tetanus is almost always recovered in areas with grazing horses from their manure and is always present in soils fertilized with large volumes of animal manure. The common and widespread occurrence of tetanus allows it to be found almost anywhere and the example of telling children they need a tetanus shot when they poke themselves with a rusty nail is probably well founded.

In the United States, C. tetani is found most widespread in the midwest, southern and southeastern states. It is found in a minimum of 20% of all soil samples and this percentage rises with proximity to cultivated lands. Despite a reduction of incidence rates of .3 to .04 cases per 100,000 population from 1950 to 1967, and the increased use of immunization, the mortality rate has remained about the same.

When washed C. tetani spores alone are injected into animals they fail to germinate and are consumed by the phagocytes with no resulting illness from tetanus. The pure cultures, washed free from toxin and injected into healthy skin tissues are unable to grow and proliferate. The germination of spores is dependent upon the reduced oxygen content of devitalized tissues and non living materials in the wound upon which it can grow. When infection does occur, it remains strictly localized at the site of original growth (the wound) and the effects of the disease are from the toxin diffusing into the neighboring tissues and spreading from there.

C. tetani spores have been carried on invisible dust to surgical instruments, directly into wounds during surgery, onto plants and common everyday objects that pierce skin such as thorns, nails, wood splinters, etc. Gunshot injuries and automobile accidents are also common sources of tetanus infections.

Following infection of the wound, burn, or injury, the tetani may incubate for 4-7 days, occasionally it may take a month before germination and toxin spread takes place. A severe headache followed by difficulty in swallowing and in opening the mouth (lockjaw) with spasms in the jaw and neck, are the earliest symptoms. Muscle spasms soon follow with generalized rigidity and convulsions as the toxin affects the entire nervous system. The spasms in some cases is so bad that bones are broken. Untreated, tetanus is almost always fatal. If the individual survives, the only lasting effects are from injuries due to strain from the spasms.

The spores of Tetani are highly resistant to many adverse conditions in nature and the lab. Most strains can be killed by boiling in water for 5-15 minutes but a few strains can survive boiling for as long as three hours. They resist dry heat at 150 C for one hour, 5% phenol and .1% mercury perchloride for up to two weeks. 1% Aqueous Iodine or hydrogen peroxide will usually kill the spores in a few hours. Tetani survives most disinfectant.

Heating at 150 C for 30 minutes and then soaking in 5% phenol for 6 hours will kill all non spore bacteria and most spores of other species. This is followed by separation and differentiation by cultural characteristics followed by injection into mice to confirm the symptoms of tetanus.

<u>Growth and Production</u>

Under the microscope, the bacilli are straight, slender rods, 2-5 um x 3-8 um with rounded ends. Some cells have shorter forms and longer filaments, are motile, with many long peritrichous flagella. Movement is not very active. The spores in early development may produce oval enlargements. The fully developed spore is terminal and spherical, two to four times the diameter of the bacillus producing a drum stick appearance which is striking feature of the organism making it easy to separate this species from other spore formers. The drum stick appearance is often used to presume the presence of tetani.

Strains vary in their ability to produce spores. Spore forming can be enhanced by including horse meat and 1% glucose to agar and is interesting that horse manure often contains tetani spores. Spores form in the late growth phases after about two days in cultures at 37 C. They are highly resistant to chemicals and physical destruction. They remain viable for years just below the surface of soil protected from sunlight and excessive heat. The spores are destroyed by 5% phenol only after at least 10-12 hours soaking.

The vegetating bacteria is killed rapidly on exposure to the oxygen in air so must be carefully transported and handled to maintain live specimens. When grown in culture the colonies have a "burnt-organic" smell.

Tetani stains gram positive but there are gram negative forms that are recovered from broth cultures. Only the periphery of mature spores will absorb the counterstain. The spore does not absorb the gram stain and appears as a colorless round structure. With prolonged incubation the vegetative cells autolyze leaving either the spore with part of the cell attached, or only free spores.

C. Tetanus grows anaerobically in a wide range of temperatures (14-43 C) with the optimum temperature of 37 C and an ideal pH of 7.4. It grows on all ordinary media but grows best in cooked meat or peptic blood broth. The addition of chopped meat and/or brain tissue enhances growth on all broth media. The broth's are usually boiled to expel absorbed oxygen and sealed with paraffin wax or petrolatum. It they are 7-12 cm deep, or if the medium is viscous, then no seal is usually needed.

On solid media, surface colonies of the normal, motile tetanus bacillus have long, branching projections. The colonies which grow to about 1 mm in diameter at 48-72 hours become slightly raised with a ground glass appearance and the edges having a delicate filamentous character. A fine spreading (swarming) rhizoidal growth may extend over the entire medium, as a thin film, usually on blood agar, that may not be easily seen and that prevents the easy recovery of isolated colonies. A plate can be inoculated on one half only. The swarming can allow recovery of the tetani because of the motility that allows it to move in advance of other organisms when condensed moisture occurs on the plate. The advancing edge that grows to the uninoculated side is taken off the plate by wire loop.

In mixed, sugar free cultures, C. tetani has grown on culture plates with aerobic organisms in contact with air because the other aerobes reduced the oxidation-reduction potential enough to permit its growth. Neosporin antibiotic may be added to the culture media to inhibit other organisms while permitting growth of C. tetani.

On blood agar, a faint hemolysis occurs in the area of initial growth and may develop below the colonies, but it rarely appears below young colonies. Non-motile colonies with isolated growth may lack these features. It is weakly proteolytic.

In glucose broth, mixed cultures often produce acid which inhibits the ability of C. tetani to grow. This is also why it does not grow in the intestinal tract of man even though its spores will survive. In pure culture glucose stimulates C. tetani growth.

On agar stab cultures, no growth occurs on the surface while a white line of growth appears along the track of the inoculating wire but stops short of the surface. Lateral spikes grow from the central spike radially with the longest in the deepest part of the tube.

Nutrient gelatin is only slowly liquefied taking more than 48 hours to be visible. Coagulated proteins such as serum becomes slowly transparent and softened. Litmus milk may show no coagulation or may result in delayed clotting. Cooked meat and deep brain mediums show slight digestion and the meat is blackened. Some gas is produced from the breakdown of amino acids and no carbohydrates are usually fermented. The cultures have a slightly pungent, unpleasant odor. Biotin and many amino acids are required in artificial mediums. Toxin production is best on complex mediums containing both beef infusion and peptones.

Samples cut out from infected wounds of animals with tetanus can be mixed with sand and ground up to a dust to yield a workable material of mixed cultures. This is direct plated on blood agar and grown anaerobically. The mix of colonies can then be swabbed into broth and heated at 80 C for 10 minutes and then recultered. The resulting subcultures can each then be used for direct inoculation into test animals.

When mixed cultures are present, the C. tetani is incubated anaerobically in 5% peptic blood broth for 2-4 days at 37 C. The culture is then heated to 80 C for 30 minutes to kill spreading, non-sporing organisms like Proteus. The condensed water from this plate is then inoculated into a test tube with the same media. There is a mass of very fine filaments and subcultures from this mass usually result in pure C. tetani.

Testing of the final culture is usually done in mice. A 5-10 day cooked meat broth culture is prepared and .2 ml is injected into the tissues to the right of the base of the tail. With a positive test, in about a day there may be stiffness in the tail and hind limbs, with the right hind leg becoming paralyzed and the tail and spine of the animal curving to the right. Tetanic convulsions soon follow. Control animals are given a tetanus shot one hour prior to the above inoculation.

<u>Toxicity and Harm</u>

In nature, tetanus usually is the result of contamination of a wound in which the C. tetani accompany the piercing instrument or debris. In war, these usually are disseminated by pungi sticks, bullets, grenades, and shrapnel from flying debris and soil. On farms, the organism can enter abrasions or cuts from the dust of dried up manure blowing in the air, or simple contact with dirty clothing.

The conditions which most favor germination and multiplication of the organism in tissues are deep puncture wounds, wounds accompanied by compression injuries which cuts off blood supply and kills living tissue in the area, necrotic tissue, effused blood, wounds contaminated with soil and foreign bodies such as pieces of clothing or bits of shrapnel. Other organisms that accompany C. tetani such as C. welchii and pyogenic cocci also aid in germination because of their effects in surrounding tissues.

The exotoxin contains a neurotoxin component that is the primary essential pathogenic constituent. It develops in broth culture after 5-14 days growth at 35 C. The toxin yields vary by culture media and strain of tetani.

There are two toxic components to the tetanus, one called a "Tetanolysin" that causes lysis of the red blood corpuscles, and "Tetanospasmin that as a pure crystalline protein kills with an LD50 for the mouse at .0000001 mg. This makes it nearly as deadly as the Botulinal and dysentery toxins, all three of which as a group, stands alone in ability to kill in tiny amounts. It is highly soluble and diffuses into and affects the nervous system directly. The toxin constitutes about 5-10% of the bacterial weight at the time of lysis when it is released which allows for very large production volume of this material in relation to the amount of culture media.

The tetanolysin disappears from grown cultures in about two days due to rapid inactivation and is not useful as a weapon.

The tetanospasmin is released from the cells as they die (autolysis) and accumulates in the culture. It has also been extracted from the living cells with molar sodium chloride solution. In water solutions the toxin is unstable to both heat and light but is stable in a dry and purified form. It occurs as a single antigenic type and makes an excellent antigen that gives rise to high titer antiserum that neutralizes the activity of the toxin. The toxin is a protein and is destroyed by the proteolytic enzymes which is why humans are not harmed when it is ingested with foodstuffs.

On release from the cell by lysis, the tetanospasmin is acted on by bacterial enzymes which alter its form to two types of deadly toxic chains, both of which have similar toxicity. The toxin is transported along the nerve endings to the spinal cord where it binds to and interferes with the release of inhibitory neurotransmitters. In this manner it is similar to strychnine. The toxin may also induce flaccid paralysis by action similar to that of the Botulinal toxin. Death is usually by respiratory failure and it may even be hard to find the original infection site.

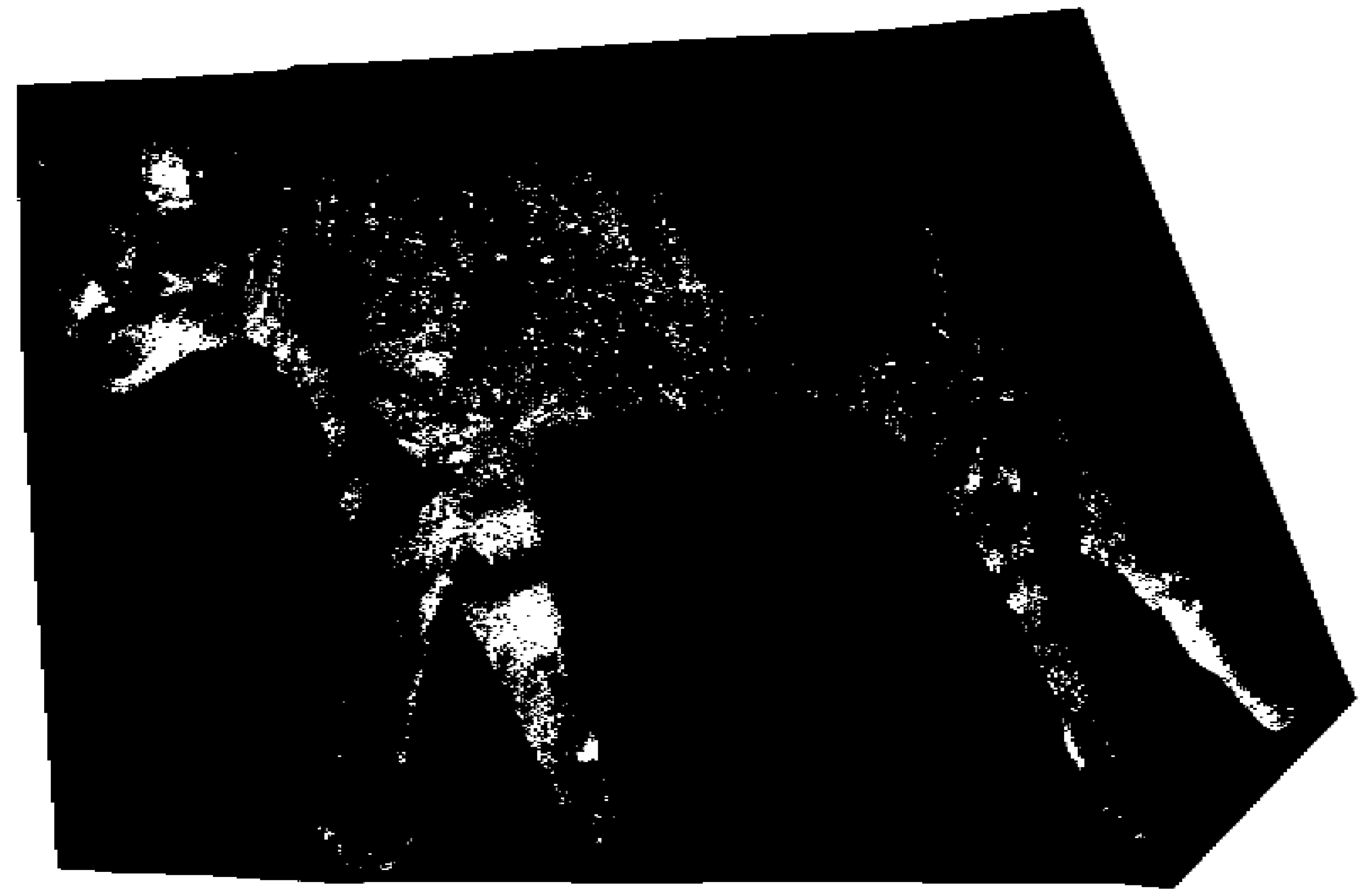
When test animals are injected with the tetanus toxin, they die within days of the classic signs of tetanus. Spasm usually start near the site of injection and pass with the toxin to the central nervous system spreading along the nerve fibers to the entire nervous system. Paralysis of the associated muscles controlled by the affected parts of the nervous system occurs until the paralysis stops breathing and the host dies.

It has been established that the main mode of action of the toxin is that it suppresses inhibition in the mammalian spinal cord. It is also believed to block neuromuscular transmission. The tetanus toxin does not travel by the bloodstream but uses the nervous system itself to reach the spinal cord where it causes its effects. When tetanus is introduced into the bloodstream as it would be in inhalation weapons it would have to travel until coming into contact with motor nerves where it would begin its travel to the central nervous system. This would cause a delay in toxic effects, depending on the dose inhaled.

When injected with washed spores with no toxin, no disease occurs unless the area is deliberately injured by bruising or lacerations to create the physical cut off of blood flow to the area. Once this is done the disease can progress. An addition of 2% calcium chloride was included in injections in mice to cause local necrosis in tissues and initiation of the tetanus disease.

A cat at 3 days and 5 days after injection with 2.5 mg of toxin.





Injection of .2 ml of seven day old C. tetani into the subcutaneous tissues at the right of the base of the tail of this mouse with the symptoms as we described earlier occuring here at 24 hours. The tail strongly curves to the right.



3. Processing on DEAE cellulose

4. Precipitating with 300 g/liter ammonium sulfate

5. Processing on G-100 Sephandex.

The pure toxin contains 15.7 % nitrogen, precipitates from solution and loses activity at a pH of 6 but is stable at a pH of 5 in the presence of glycine.The tetanospasmin can be destroyed by heat (65 C) but is oxygen stable.

<u>Protection by Antibiotics, Vaccines, and Natural Resistance</u>

Contracting tetanus and surviving it does not confer immunity to the individual because the amount of toxin necessary to cause illness and kill is so small that it is not immunogenic. Recurrent attacks are not uncommon among unimmunized individuals.

The tetanus bacillus possesses a variety of cellular (O) and flagellar (H) and spore antigens. It has limited value however since the bacillus usually is not in contact with living tissues when it is producing toxin and causing harm. The neurotoxin produced by all 10 strains has a single immunological type and since this is the material that diffuses into the bloodstream and tissues causing harm, this is the most important antigenic material.

Tetanus antitoxin or serum (ATS) is obtained by immunizing horses with toxoid. The serum, if given immediately after wounding, has some value, while its use as a curative after symptoms have begun is limited.

Once toxin has reached the central nervous system, it is not neutralized by circulating antitoxin. This means that the toxin can still harm even after antitoxin has been administered if it has had time to reach the nerve endings and bind to the receptor cells. The antitoxin only neutralizes uncombined toxin.

Tetanus toxoid is formed with formaldehyde and the antigen is prepared by absorbing purified tetanus toxoid on aluminum hydroxide. This is released subsequently in the body over a period of days.

In superficial field wounds, cleaning and proper draining is usually sufficient. With deep wounds and stabs, bruising with punctures, and animal bites, a program is undertaken to treat the individual when infection is certain. This involves -

1. IV injection of antitoxin followed by intramuscular injections at half hour intervals. Antiserum may also be used. Test injections are used if an allergy is suspected.

2. Penicillin is usually given when evidence of infection is observed, and is maintained until healing is observed. If allergic then tetracycline or clindamycin are used.

3. A course of immunization of .5 ml of tetanus toxoid, followed by a second shot in 6-12 weeks followed by a booster at 6-12 months has been used. An absorbed preparation of high concentration of toxoid may require only one shot.

4. The mild spasms may be controlled with barbiturates and diazepam. For severe spasms a curare type agent may be employed to paralyze the patients muscles while respiratory function is maintained by positive pressure breathing apparatus. Auditory and visual stimuli are also reduced.

The patient is regarded as immune for 6 months following the first two injections and for 5 years following the third injection. Antitoxin is not usually given to immunized individuals while a booster shot of toxoid is the preferred method of treatment.

It has also been found that increasing oxygen and pressure (hyperbaric) in the blood and tissues of humans with tetanus slows and sometimes arrests the progression of the disease.

Even with antibiotics, immunization, and all other methods of treatment, mortality rates for tetanus still reach 30-50% once the infection takes hold..

Incorporation into Weapons

* The definition of MLD (minimum lethal doses) needs clarification in this chapter. It is not always clear how this is defined by the different researchers. Some regard it as the least dose that will kill the animals when injected. Does this mean all, some , or one animal. In actual tests, the LD 100 (lethal dose to kill 100% of the animals) is 1.4 times that of the LD 50 and 1.7 times that of the LD 0 in which none of the test animals died. Of these numbers, the LD 50 is used as the most meaningful since it lies on the steepest part of the dose response curve. The LD 50 should also include the time frame in which the animals died (such as 4-7 days).

Tests on mice receiving 1 MLD by injection required about 96 hours to die with all the classic symptoms observed. At 500,000 MLD death occurred in 60-70 minutes without many of the clinical symptoms. It is possible to achieve 1,000 MLD human dosage on single invisible dust particles with nearly pure tetanus toxin at sizes of less than 5 microns. This dosage will also greatly exceed that which is required to overcome the tetanus vaccines immunity.

Clostridium Welchii (Perfringens)

History and Recovery from Nature

This is the organism most commonly associated with gas gangrene. Gangrene is characterized by rapidly spreading oedema, necrosis, and gas production. Like tetanus, the infection begins in subcutaneous tissues where a physical injury has implanted the organisms and tissue fluids accumulate with extravasation of blood. The wounds have a typical foul odor and show evidence of gas formation.

It was first isolated in 1891 by Achalme and was given the name C. welchii in England while American workers named it C. perfringens.

In WW1 and WW2, Clostridium Welchii was recovered from 60% of all the wound tissue and fluid samples. The same predisposing factors necessary for C. tetani also apply here. The wound must cut off flow of oxygen to the tissues. The organisms do not travel beyond the original site, instead growing on devitalized tissues and producing toxin which diffuses into the surrounding area.

In deep wounds where the blood supply is cut off and Clostridia are introduced from the soil, shrapnel, and clothing fragments, reduced oxygen tension occurs. Lactic acid accumulates resulting in a pH drop. This activates proteolytic enzymes which attack and dissolve the tissue. These tissues are now in a form where they provide abundant food for the Clostridia. In combination with the low oxygen the bacillus grow and release toxin into the surrounding tissues. The toxins destroy and solubulize the tissues allowing the spread of the organism to proceed into the new areas. Edema fluid and gas accumulates creating pressure that cuts off blood flow which extends the area for growth and causes great pain.

The bacilli are most often found in nature in the solid waste of man and animals and is considered part of the normal intestinal flora. Its occurrence in the large intestine of man and animals approaches 100% allowing for its easy recovery from dead animals or solid wastes. Type A is also abundant in soils as both spores and vegetating cells. It often invades the blood ante-mortem and multiplies in the internal organs after death producing the observed gas cavities at autopsy. It sometimes occurs in uterine infections and is spread by the blood stream producing intravascular haemolysis.

In pig intestines, the normal cell count is 250 per gram. When fed on a very high protein diet, these numbers may increase to more than one million per gram resulting in enterotoxemia. Similar effects have been observed in dietary changes of other species. All depend on an abundant supply of carbohydrates.

C. welchii are broken down into five different types. These types are distinguished by the combination of toxins they produce. The classical gas gangrene is caused by type A which is by far the most widespread while the others are more commonly associated with disease in animals.

These toxins diffuse into the surrounding tissues as they are produced and act as enzymes breaking apart the living tissues leaving behind dead (necrotic), rotting matter. They continue to move into the surrounding areas and usually require amputation of affected areas to save the life of the host.

The lethal, lecithinase, and hot-cold haemolysis activity of the C. welchii are all the result of a single alpha toxin. *The hot- cold haemolysis means that if you incubate the C. welchii and it produces alpha toxin on erythrocytes (blood), no haemolysis takes place at 37 C, but when the suspension is cooled the erythrocytes hemolyze. This is one way to tell them apart from other organisms.

Food poisoning strains are also widely distributed and its incidence usually occurs when a pre-cooked meat food has been allowed to stand at near body temperature allowing the C. welchii to reproduce within the tissues inside. These spores of these strains are heat resistant but usually only reproduce modestly and only cause cramps followed by diarrhea. Symptoms usually subside in 24-48 hours.

C. welchii can almost always be recovered by taking a swab specimen of feces and stirring it into a cooked meat broth and heat it at 50 C for an hour. It is then incubated overnight at 37 C. Many organisms will likely be recovered producing a mixed culture which must be separated out and tested from plated media.

The infection of C. welchii yields gas gangrene with massive necrotizing of muscle, severe shock, and always results in death in untreated cases.

Growth and Production

C. welchii is a relatively large gram positive member of the bacillus genus about 4-6 *u*m x 1 *u*m, with square or rounded ends and occurring singly or in pairs or chains. It is non motile with spores that are typically oval, subterminal and non bulging. It is often encapsulated when seen in tissues. When grown in a sugar media the bacilli are shorter while in protein media they become filamentous. During rapid growth they produce many short forms, some which are almost coccal in appearance.

Spores can be grown only in the absence of fermentable carbohydrates. They are subterminal and do not swell the vegetative cell in which they are formed. The spores can assume odd forms and even the same culture can be variable in microscopic appearance. C. welchii is an obligatory anaerobe that grows best at 37 C on carbohydrate containing medium such as glucose or glucose-blood agars or gels. It grows readily on deep brain and meat infusion media's which do not blacken but will discolor when metallic iron is added which aids in identification. It grows in a pH range of 5.5 to 8 and from 20-50 C.

welchii. Heat treatment should not be used to isolate the organism because the most virulent strains appear heat sensitive.

It is advantageous to add Neosporin to the medium to restrict the growth of gram positive organisms. Use of partial anaerobic conditions will prevent the growth of other swarming Clostridia like C. tetani. This can be done by reducing the air in the container under vacuum to -600mm Hg and replacing it with hydrogen.

Surface colonies are large, smooth, regular, convex, with semitranslucent, slightly opaque discs but other forms are also observed. One has a raised opaque center with a flat transparent border which is radially striated. Some have rough flat colonies with an irregular edge resembling a vine leaf. On horse blood agar, the colonies produce a zone of variable complete haemolysis with a wider zone of incomplete haemolysis occasionally occurring. Sometimes a mucoid form from a broth culture will produce very tenacious colonies on blood agar.

As colonies age the periphery may lose symmetry and look like a swarming motile colony instead. On blood plates, type A can usually be identified by a narrow zone of complete hemolysis due to the theta toxin and a much wider zone of incomplete hemolysis due to the alpha toxin. This double zone may fade with incubation longer than 12 hours. Adding .5% calcium chloride to the media aids in double zone formation by making the cells more susceptible to the action of the alpha toxin.

In litmus milk, all strains of the C. welchii produce acid, clotting, and gas production. The gas breaks up the clot producing the "stormy clot" reaction. The culture has a sour, butyric acid odor. It ferments glucose, lactose, sucrose, maltose, and starch with gas production. Some strains ferment salicin, glycerol, and inulin. Mannitol and dulcitol are not fermented.

C. welchii liquefies gelatin but not coagulated serum. Cooked meat is reddened or pink, but no digestion occurs. The type A spores are resistant to almost all disinfectants and antiseptics but have only modest heat resistance and die off at boiling for only a few minutes. The spores of type C and some food poisoning strains may be very heat resistant and can survive boiling for hours.

Using egg yolk medium to identify those colonies that produce an opalescence is one way to separate and identify this species. All clostridia that produce lecithinase enzymes will yield a zone of opalescence on egg yolk (5%) or human serum in agar or gel. Neosporin may be added to inhibit other aerobic sporing organisms and coliforms. Adding lactose and neutral red will help identify its lactose fermenting while adding milk results in proteolysis.

Toxin production is increased by using polymers of glucose such as dextrin, starch, and glycogen in the medium. Peptides and peptones instead of free amino acids also increases toxin yields and the larger the molecules the better. During production, the pH drops to 5.5 because of the co-production of free acids which stops toxin formation. The pH of the medium must be maintained and adjusted periodically to 6.7 - 7.5 for maximum yields. The release of toxic protein from the intact cells is pH dependent.

Toxicity and Harm

There are different combinations of toxins and enzymic factors produced by C. welchii. Some of these have haemolytic, lethal, or necrotizing properties while others use enzymatic activity to attack tissues.

Strain Type	Toxin produced
A	alpha, delta, eta, kappa, mu, nu
B	alpha, beta, epsilon, theta, gamma, eta, lambda, mu, nu
C	alpha, beta, theta, gamma, eta, kappa, nu
D	alpha, epsilon, eta, kappa, mu. nu
E	alpha, iota, eta, kappa, lambda, mu, nu

Alpha toxin is produced by all types of C. welchii, but most notably by type A strains. This is the main cause of the profound toxemia associated with gas gangrene in man. This toxin is lethal in lab animals and it is necrotizing on intradermal inoculation. It is fairly heat stable and is about 50% inactivated after 5 minutes at 100 C. The toxin is the enzyme, lecithinase C. As long as free alkali ions, specifically calcium or magnesium are present, it can split lipoproteins complexes in egg yolk or serum agars and gels with the opalescent ring around the colony. This reaction is inhibited by the specific antitoxin.

The lecithinase also attacks phospho-lipid components in red blood cells resulting in haemolysis (clear zones) around colonies on the blood of most animals except the horse and goat. When a zone is produced around horse blood with Type A C. welchii it is because of the theta toxin and not the alpha toxin. The alpha toxin destroys erythrocytes, leukocytes, and platelets as well as weakening the muscle cell plasma membranes.

The lecithinase C is actually phosphatidase enzyme which hydrolyzes (splits it in two) lecithin at the C bond which is how it was named. The two parts left over are phosphorylcholine and diglyceride. It is different from snake venom lecithinase A which requires activation by lecithin and cephalin. The optimal pH for effectiveness of this enzyme is 7.4-7.6 in borate buffer to 6.7 pH with bicarbonate and carbon dioxide buffer. This recommends the addition of bicarbonate on dust weapons to maximize effect.

Calcium is also required for lecithinase activation and the addition of calcium chloride (.1 mole) not only activates it but assists it in producing localized necrosis when added in small amounts. Magnesium, zinc, cobalt, and manganese all activate the enzyme as well and are essential for its activity. Lecithinase C is stable and withstands heating at 100 C for 10-15 minutes with 45% activity. Egg albumin is used to stabilize and store the enzyme. Saline will inactivate the enzyme.

The erythrocytes of sheep are poor in lecithin, however the erythrocytes in the horse, rabbit, and man are rich in lecithin and this accounts for its greater effect (hemolysis) in human tissues and serum.

This toxin can be purified by using ammonium sulfate fractionation and nucleic acid precipitation at pH of 4.5 with subsequent precipitation with protamine. It has also been separated by fractionation in methanol-water mixture in 4 steps. These methods increase the purity by 130-250 fold while separating out cellular matter, proteins, and other toxins.

When alpha toxin is injected into animal bloodstreams, it is rapidly cleared from the circulatory system and is found in large amounts in the liver, kidneys, lungs, and spleen. The toxin is rapidly metabolized and breakdown products are excreted in the urine and partially eliminated as CO2.

Beta toxin is produced by types B and C. It is also lethal and necrotizing. Once inoculated into guinea pigs, a circular purple-tinged necrotic area occurs. In Type B strains which produce a hyaluronidase, the area may be more extensive and irregular. When injected into animals bloodstreams it causes profound changes in blood pressure which may lead to heart failure.

This toxin damages intestinal villi permitting attachment of C. perfringens and helps bring about necrosis of the gut wall during intestinal infections.

Epsilon toxin is produced by type B and D strains. It is a prototoxin which must be activated by proteolytic enzymes. It is lethal and necrotizing and depends on an alkaline environment with the activating enzyme to become deadly. It is very toxic to sheep and is converted to active toxin by proteolytic enzymes action. It exerts a neurotoxic effect when distributed by the bloodstream (sheep). It also systematically destroys the kidneys. It has also been shown that it increases permeability of the small intestine allowing the uptake of itself and other toxins to be considerably increased. This commends its use in combination with ingestion based weapons such as the Botulinal toxin.

Toxin is purified by methanol fractionation in only very small yields that limit its study. It is activated by the addition of trypsin to the media. Even in its inactive form, if it is injected into the animal it will be activated and will produce the toxic reactions and death with a 24-48 hour delay.

This toxin is much lower in lethal effect than the Botulinal toxin. For comparison, the Botulinal has an MLD of 2.7 billion per mg of nitrogen (mice) while crystalline trypsin activated Epsilon toxin has a value of 30 million per mg of nitrogen.

This toxin produces the following effects -

1. Increases vascular permeability allowing rapid uptake of itself and other toxins from the intestine.
2. Accumulates in the tissues, particularly in the brain.
3. Produces edema in the nervous tissue.
4. Forms necrosis of the nervous tissues.

Iota toxin is produced only by type E strains and is a prototoxin that requires activation like the epsilon strain. This is accomplished by proteolytic enzymes as well resulting in a lethal necrotizing effect. The trypsin activated toxin yields about 150 LD50 per ml in liquid culture. This is a 100 fold increase in toxicity over inactivated toxin.

Iota toxin injected into guinea pigs results in a drastic increase in capillary permeability which results in blue areas developing around the injection site. This increases the diffusion rate and spread of toxin throughout tissues and the body.

Theta toxin is an oxygen labile heamolysin that is produced by most strains of C. welchii but not those that produce food poisoning. It lyses the red blood cells of horse, ox, sheep, and rabbit but does not affect the mouse erythrocytes. It is known that tissue lipids and cholesterol inactivate theta toxin as well as oxygen in the air. Once inactivated by air it can be reactivated by reducing agents such as cysteine, thioglycollic acid, and thioacetic acid. It is absorbed into meat particles and deactivated and must be grown in meat free medium to recover this toxin. It is considered lethal because it lyses blood cells.

This toxin is destroyed by heating at 60 C for 30 minutes and is significantly inactivated at 37 C. It is stable in cold temperatures. This hemolysin destroys the erythrocytes of man, pig, dog, cow, sheep, horse, mouse, and rat. It produces hemolysis on the erythrocytes of rabbit, guinea pigs, chicken, and pigeon. It is readily absorbed onto the erythrocytes and on filters.

It also causes lung edema in rats and this may be useful in inhalation weapons. The effect of IV injections on the lungs were so great in causing edema in the rats that they quit breathing while their hearts were still beating.

Gamma toxin is a minor lethal toxin.

Delta toxin is haemolytic for the red cells of even toed ungulates (sheep, goats, pigs, and cattle) and is lethal. It is reversibly activated by certain reducing agents such as sodium hydrosulfite, thiolacetic acid, or cystein. It is serologically related to streptolysin O and tetanolysin. It is dermonecrotizing and does not require alkali ions for its activity.

It is separated by fractionation in methanol water mixtures and exhibited no collagenase, hyaluronidase, and contained less than .01% of total lecithinase activity of the parent filtrate.

Eta toxin is considered an insignificant lethal toxin.

Kappa toxin is a collagenase enzyme which also attacks hide powder and gelatin as well as native collagen. This is the toxin that causes the softening of muscle connective tissue in gas gangrene. When pieces of animal and human tissues are incubated in Type A filtrates, they disintegrated to a mass of discrete muscle cells and the collagen from various mammal tendons was also dissolved.

[The collagenase activity produced by Cl. histolyticum is at least 10 times more active and more useful as a weapon. It makes a good combination weapon which is what is observed when many different organisms are recovered in gas gangrene cases.]

The toxin was first purified by carefully controlled absorption on calcium phosphate followed by elution with dilute ammonium sulfate, and last by fractionation with ammonium sulfate. The precipitate is dissolved in water and 1 mole calcium chloride, and .05 mole phosphate buffer (pH 6.8) were then added. The resulting precipitate of calcium phosphate absorbed the collagenase. It was eluted from the calcium phosphate with a 10% solution of ammonium sulfate. It was further freed from pigment by treatment with activated charcoal.

It can also be isolated by precipitation with methanol at pH of 9.0 with the final purification carried out on a calcium phosphate column.

When injected intramuscularly in mice or guinea pigs a local swelling occurs that lasts about two days. On intravenous injection (.2 mg), massive lung hemorrhages occur, and with intracutaneous injection there is local hemorrhage and necrosis.

Lambda toxin is a proteinase and gelatinase that decomposes hide powder but not collagen. It can also destroy the alpha toxin when stored together.

Mu toxin is a hyaluronidase. It hydrolyzes hyaluronic acid that is essential in connective tissues, vitreous body, and umbilical cord of the human, and the byproduct sugars produced are fermentable by C. welchii. Its presence drastically increases the diffusion of all toxins into the surrounding tissues and increases or aids additional infection spread from other bacteria and viruses.

It has been observed that only the most virulent strains produce Mu toxin which aids in their ability to spread growth and toxin into the surrounding tissues and overcome host defenses.

Mu toxin is recovered from filtrates by precipitation with ammonium sulfate and subsequent absorption of the concentrate on aluminum hydroxide. Ammonium sulfate and ethyl alcohol at pH of 9.0, 7.5, and 6.8 were used to produce purification as well.

Nu toxin is a deoxyribonuclease. It is a prototoxin that is activated by trypsin. When injected intracutaneously in moderate amounts, it increased the permeability of small blood vessels of the skin of guinea pigs. Slightly larger doses produced necrosis. It directly destroys the connective tissue cells and nuclei of the muscle.

The recovery of toxin does not depend on lysis of the bacterial cells. When mediums contain glucose, the toxins are liberated and more easily diffused out of the cells. Once spores are formed, the spores are rich in lecithinase activity. Nu toxin is secreted into the medium during the log growth phase. This type of toxin is also present in diphtheria and Staph infections.

The presence of this toxin aids in the spread of the other toxins and organism growth. In other words, it increases its virulence. This once again demonstrates the important role of combination organisms and toxins that combine their effects on target tissues to provide broken down tissues on which to feed and grow.

[Authors comment- It should be possible to produce a dust weapon containing enzyme and nutrient combinations that will allow anthrax and other aerobic infective organisms to germinate and grow directly on exposed skin tissues. In the earlier section on Anthrax, it was recorded that large numbers of spores were recovered on the skin and in the hair follicles of the nose without creating infection. This was because the skin tissues and respiratory tract lining tissues will not support the germination, pH, moisture, and other necessary growth requirements of the organism. When these types of enzyme toxins are added with germination proteins, they can break down the tissues to provide fermentable media on which to germinate and grow.

These types of enzyme combinations produced by C. welchii and other species may make that possible. That means that a toxin combination based dust can directly infect on skin contact, or can easily infect large portions of the respiratory tract that would otherwise be immune from infection. Additives to draw moisture from the air could be added to moisturize skin that is too dry to sustain organism growth, or enzymes might be added that would liberate molecules of water to provide the moisture needed.]

Recovery and purification of mixed toxins has also been accomplished by ultrafiltration under pressure and electrophoresis on ethanol cellulose for 18 hours at 1,000 volts and 78 mA. The yields for all components were very high without being separated. This yielded nine different antigenic components. They can also be partially purified in mixed toxins by precipitation with ammonium sulfate and/or organic solvents (alcohol).

In 1962, Soviet researchers used precipitation with sodium hexametaphosphate for toxin purification and obtained activity of 275 Lf/mg of total nitrogen [Lf=lethal factors].

When toxins are produced in mixtures, some are broken down by the action of others. It is necessary to store the toxin mixes above a pH of 8 for stability.

Some strains of C. welchii cultures possess other enzymatic properties which destroy blood group substances. The organism also renders red blood cells inagglutinable to the myxovuruses due to a receptor destroying enzyme (neuraminidase) similar to that found in cholera.

It is possible to take some strains into prolonged subculture and get them to eventually produce a diffusable haemagglutinin which causes agglutination of the red blood cells of man and most animals. It is not produced by fresh strains.

Toxin rate of production reaches a maximum within 4-5 hours after the bacteria begins to grow. It first appears at 4 hours and peaks at 8 hours growth. The greatest production of alpha toxin occurred on meat-peptone bouillon containing .15% agar. The addition of .5% glucose stimulated production of the lecithinase and hyaluronidase factors. Toxin production generally decreases at 12 hours. The peak period of toxic lethality (potency) occurs at 6-9 hours and declines afterwards as the continued growth of the bacillus reduces the toxin potency. This has been confirmed in animal injection tests. Maximal toxin production and potency occurs during the period of most active cell division.

The toxins are all proteins even though some are enzymatic in activity. The toxins are synthesized and released from parent cells during growth making them true exotoxins.

Testing in animals to confirm the organism is done by injecting a guinea pig in the right hind leg intramuscularly with 1 ml of a fresh 24 hour cooked meat broth culture. In the first few hours a marked swelling occurs in the injected limb from the gas formation. The oedema spreads up over the abdomen. Death occurs in 24-48 hours.

<u>Protection by vaccination, antibiotics, etc.</u>

Treating the toxins for 48 hours with formaldehyde, harmless toxoids with high immunogenic activity are produced. They have had very limited effectiveness in actual use however. When the infection actually starts, the blood flow is cut off and does not reach the affected area with antibodies.

Antitoxin theoretically neutralizes the major lethal antigens of these types -

Type A antitoxin	only homologous toxin
Type B "	Types A, B, C, and D toxin
Type C "	Types A and C toxin
Type D "	Types A and D

The epsilon and iota toxins do not occur in fully active form in cultures and their prototoxins require "trypsinisation" to activate them. This is accomplished by adding .005% crystalline trypsin to the crude filtrate for one hour at 37 C.

All cases of gas gangrene produce 100% fatalities unless the infected muscles have been amputated. Even when given enormous doses of antibiotics and antitoxin, the infected tissue must be removed to prevent death.

Antitoxin therapy is only useful if given in the first stages of intoxication <u>and</u> accompanied by amputation. No preparation of toxoid or immunization has been proven protective.

The reasons for the inability to immunize is because 1) most of the toxins are enzymes which, as a rule are weak antigens, 2) It is necessary to produce strong antitoxic immunity, 3) the toxins are found in combinations of lethal enzymatic, hemolysis, and lecithinase making the production of immunological materials difficult and complex because the components that produce each of these activities have not been immunologically separated.

All types of toxins in the C. welchii total 12, 5 of which are enzymes. The toxins also contain neuramidase which inhibits receptor function of cells of the host. This multiple attack further complicates providing protection.

The use of hyperbaric oxygen to raise tissue oxygen has reduced fatalities about 50%. The patient is given 100% O2 in a pressurized chamber at 3 atmospheres. This is done three times in the first 24 hours and four more times at 6-12 hour intervals.

Antibiotics have little effect although they are used as ancillary agents. Penicillin, klindamycin, gentamycin, and kanamycin have all been used. Surgical removal of the affected areas is usually the only way to prevent death.

Clostridium Novyi (Oedematiens)

History and Recovery from Nature

C. Novyi, is a gram positive anaerobic bacillus with cells appearing thick and rectangular with rounded ends, 5-10 *u*m x 1 *u*m and resembles C. welchii in colony morphology but is somewhat larger and pleomorphic. It it not motile when examined microscopically because of its sensitivity to air, but does freely produce spores with peritrichous flagella in nonfermentable mediums. Its spores are oval, central or sub-terminal. The vegetating cells die rapidly on exposure to air. Young cultures resemble C. welchii.

It was first isolated and described by Novy in 1894 as a cause of animal infection. C. novyi is the name used by the Americans with C. oedematiens used in Europe.

Four types of C. novyi have been identified and designated A, B, C, and D. The A type is found widespread in soils, and both type A and B in the livers of many, apparently healthy animals. Type B cells are physically larger than type A under the microscope. When grown on blood agar they quickly swarm across the plate. Under the microscope, the flagella become entangled in "bouquets" which aids in early identification.

Media are cultured anaerobically with a dry surface to slow spread of the colonies during growth. The agar or gel should be increased to 4-7% to inhibit spreading. Add egg yolk to identify the lecithinase and lipase reactions (opalescent underneath with an overlying iridescent pearly layer which seems to indicate corresponding lipase activity), and neomycin (Neosporin) to inhibit gram positive organisms.

Surface colonies grown on thick agar or gel produce an irregular, circular, 2-3 mm diameter, semitransclucent, finely lobated or crenated edge appearance. They are extremely delicate, flat, and bluish gray in color.

Cooked meat should be inoculated and heated to 100 C for 5-10 minutes to kill non-sporing bacteria. C. novyi grows abundantly, especially with fermentable sugar. In subculture, the plates are incubated at 37 C and examined at 24-48 hours.

A zone of *B*-hemolysis coextensive with the colony is produced on horse blood agar.

Growth and Production

Surface colonies are transparent, flat, irregular and tend to fuse together forming a spreading growth or film. Blood agar cultures produce slight hemolysis. The very small "daughter" colonies may move on the surface of the agar and break away from the edge of the parent colony. Colonies on heated blood agar may produce a green halo (bleaching) and if benzidine is added to the medium they will blacken after being exposed to air for an hour which aids in identification.

Some strains will not grow on solid media unless freshly prepared and the addition of fresh brain infusion aids in growth of these fastidious organisms. Those strains that do not grow on the surface of these mediums may be grown in deep media or gel that is shaken to spread the spores. These colonies appear as small, irregular, woolly, or snow flake-like balls of growth. The media is prepared by mixing the specimen in a broth and adding it to melted agar at 45 C or gel prior to refrigeration. This usually allows for anaerobic growth and separation of the colonies so they can be observed and identified. For production of pure strains, cooked meat broth gives good growth with slight gas production in 24 hours (48 hours with type D). The finicky strains can grow better with cysteine and dithiothreitol added to the mediums.

C. novyi may not digest litmus milk but can clot it after standing. It ferments glucose and maltose. It liquefies gelatin, does not digest coagulated serum, and reddens cooked meat without digesting it.

There are four different serological types with different combinations of soluble antigens designated A, B, C, and D. Type A produces a pearly layer or iridescent film on egg yolk caused by its epsilon toxin with the others producing only the diffuse opalescence. Type's B, C, and D are extremely fastidious and oxygen sensitive requiring the special treatment described earlier.

Types A and B ferment glucose and maltose while types C and D ferment glucose only. All types produce gelatinase but do not attack more complex proteins. Hydrogen sulfide and indole is produced by type D strains.

Culture filtrates are very toxic producing hemolysis and lecithinase activity in addition to necrotizing and lethal effects. This allow most C. novyi to infect a wide range of animals. Type C strains are not toxic to humans.

<u>Toxicity and Harm</u>

C. novyi is recovered from the most toxic forms of gas gangrene seen in man. It causes gangrene infections in most animals as well.

Alpha toxin is produced by both type A and B and is necrotizing, and lethal and is the most abundant toxin recovered from C. novyi filtrates. The toxin increases capillary permeability and produces an intense gelatinous edema in muscle tissue with resulting death of the test animals.

Gamma toxin is a lecithinase C enzyme found in type A organisms which is hemolytic lecithinase C, and necrotizing. Produces opalescence in egg yolk emulsions at 37 C and are hot cold hemolysins (no zone hot, hemolysis develops on cooling).

Beta toxin is also a lecithinase C enzyme found in type B C. novyi and is hemolytic, necrotizing and lethal to humans. Produces opalescence in egg yolk emulsions at 37 C and are hot cold hemolysins (no zone hot, hemolysis develops on cooling). Produced in large volume by type D strains. Its activity is very strong against most mammal erythrocytes.

Epsilon toxin causes harm by the action of a lipase enzyme produced only by type A strains which gives an opalescence in egg yolk emulsions and pearly layer effect (like oil on water) on and around colonies on media with egg yolk. In test tubes, the opalescence develops after the tubes have been cooled to room temperature.

Theta toxin produces opalescence in egg yolk mediums and is made by type D and B strains.

Name	Toxin activity	A	B	C	D
alpha	Necrotizing, lethal	x	x		
beta	Hemolytic, necrotizing, lethal, lecithinase		x		x
gamma	Hemolytic, necrotizing, lecithinase	x			
delta	Oxygen labile hemolysin	x			
epsilon	Lipase (pearly layer-egg yolk)	x			
zeta	Hemolysin		x		
eta	Tropomyosinase		x		x
theta	Opalescence in egg yolk		x		x

Horse meat in the growth medium produces good yields of all the toxins.

Type D strains produce such large volumes of toxin when grown in fluid media like cooked meat broth and is so potent that when subcultured on agar, they may make a zone of both hemolysis and lecithinase without the organism even growing to produce any more.

Confirmation of C. novyi in animals is accomplished by inoculating a pig intramuscularly in the right hind leg with 1 ml. of fresh, 24 hour cooked meat broth culture. It may be necessary to add 1% calcium chloride to initiate the infection. Death occurs in 24-48 hours with profound toxemia and a spreading, massive oedema from the site of inoculation. There is a thick, gelatinous, usually colorless oedema extending from the inoculated limb up over the abdomen. The inoculated muscles are red and softened. There is no gas formation. The organism can be recovered and cultured from the heart blood and spleen. The bladder may be filled with hemoglobin containing urine. There is notably less tissue destruction with C. novyi than with other Clostridia.

If a mouse is used instead of a guinea pig, the oedema is so extensive that it assumes a flattened pear shape with its pelvic area twice its normal width. One way of separating C. novyi from other infective strains that are mixed into culture is to directly inject the mixed culture into the guinea pig with 1% calcium chloride. The C. novyi is the only Clostridia to migrate to the heart and spleen and can be recovered there.

Protection by antibiotics and immunology

There are four different serological types - A, B, C, and D which produce 8 different soluble antigens. Antitoxin and antiserum has been developed and are used in cases of gas gangrene. [Type D has also become known as Clostridium heamolyticum]

Tetracycline followed by penicillin and chloramphenicol are the most commonly used and effective (in this order) antibiotics in C. novyi infections. Both the tetracycline and penicillin have been used together with better effect. Administration is at six hour intervals with 400,000 units each of procaine and potassium penicillin.

Incorporation into Weapons

C. novyi infections in gas gangrene accounts for the highest casualty rates of the Clostridia group. It produces large amounts of edema fluid with little or no gas in the infected tissues. Because of its ability to enhance the injury caused by other Clostridia and its ability to spread to the heart and spleen, it is ideal to use it in combination weapons with the other species already described.

It is most important to incorporate a starting necrotizing or clotting chemical, toxin, or bacteria to help initiate the infectious process.

Clostridium Septicum (Vibrion septique)

History and Recovery From Nature

Clostridium septicum is often found as part of the gas gangrene group of colonizing microorganisms. It produces a malignant oedema after wound infection in cattle and sheep as well as an acute fatal disease of yearling sheep which is characterized by a hemorrhagic inflammatory lesion of the abomasum. The sheep infection begins when the organism invades the mucous membrane of the abomasum where it multiplies rapidly and kills from the associated toxemia. It is generally confined to lambs born in the previous spring and outwintered in late autumn or winter on grassy hills or lowland pastures.

It was first isolated in 1877 by Pasteur who called it "vibrion septiqe". It was also isolated and reported by Koch in 1881. It is widely distributed in nature and in the intestinal tract of man and animals from which it can be recovered and cultured via the fecal matter. The organism often invades the intestinal tract and other tissues of humans shortly after death and sometimes in living patients. It occasionally produces localized necrosis under skin cancers.

C. septicum is also common in gas gangrene in pigs and found in blackleg of cattle and sheep and is recovered in 5-20% of gas gangrene cases in humans. Its spores are primarily found in soil with livestock manure prevalent such as pastures or hog yards.

The C. septicum is sometimes regarded as two types or two species. Type A causing disease in both man and animals and Type B, also known as Cl. chauvoei or Cl. feseri causing disease only in animals.

Growth and Production

It is a large bacillus spindle shaped rod, 3-6 *u*m x .6-1 *u*m with parallel sides, rounded ends and peritrichous flagella making it motile. It also grows into long curved filaments and in the tissues it develops large, swollen, gram positive navicular forms and "citron bodies" [From the citron trees- resembling a large round fruit], especially when grown on serum or fresh tissue. Spores are easily produced and appear oval, central or subterminal, and bulging, distending the organism prior to their release. Spore production is inhibited when mediums contain excessive amounts of fermentable carbohydrates. Degenerated forms may stain gram negative. They occur singly or in pairs in type B while type A tends to form long jointed filaments.

C. septicum grows strictly anaerobically and optimally at 37 C. It grows on ordinary media with added glucose increasing its growth rate. Deep brain and tissue mediums work best. Type A surface colonies are irregular, transparent, droplet-like, and turn grayish and opaque as they age. The colonies are often small and discreet with a coarsely rhizoidal edge. They may project radial growth like C. tetani which is much thicker and has a coarse irregular surface after about 24 hours and completely covers the plate by 48 hours.

They produce complete hemolysis on blood agar. Agar stabs yield a white line of growth with short, lateral spreading. Type B is more fastidious and growth is enhanced by the addition of liver extract and are about 1-2 mm in diameter after 48 hours incubation. They are commonly umbonate, irregularly circular, shiny and semitransclucent.

Aerobic contaminants can be inhibited by the addition of Neosporin to the medium as well as heating of the culture (100 C for 30 minutes) to kill non spores.

It produces a small amount of acid in Litmus milk which will slowly clot. It ferments glucose, lactose, maltose, sometimes sucrose, and never mannitol. It liquefies gelatin but not coagulated serum. Cooked meat is reddened or pink, but not digested with good growth for both types at 48 hours with slight gas production. Type A ferments salacin and type B does not.

On horse blood agar type A produces beta hemolysis while type B normally does not on horse blood but will on sheep's blood. Both organisms produce hydrogen sulphide and neither produce indole. Both also produce deoxyribonuclease and gelatinase enzymes. The major by products of metabolism are acetic and butyric acids with some formic acid.

Toxicity and Harm

C. septicum produces three exotoxins - an alpha toxin, beta toxin, delta toxin, and gamma toxin.

Alpha toxin is an exotoxin with lethal, necrotizing, hemolytic, and possibly leukocidic properties. When injected into mice they go into convulsions followed quickly by paralysis and death. Autopsy shows intense capillary engorgement and interstitial hemorrhages in the heart. It produces a gelatinous edema and local necrosis in the tissues. It produces hemolysis on both horse and sheep blood. It as an oxygen stable hemolysin which requires 5-60 minutes before lysis of the red blood cells occurs.

Type B produces a separate **Alpha** toxin that is an oxygen stable necrotizing hemolysin. It is unrelated to type A alpha toxin.

Beta toxin is a deoxyribonuclease produced by both types.

Gamma toxin is a hyaluronidase produced by both types.

Type A organisms also produce another oxygen-labile hemolysin that is similar in properties and antigenically related to this toxin. This toxin requires no induction period to cause lysis of erythrocytes. This toxin is called **Delta** toxin.

A fibrinolysin, neuramidase, and hemogglutinin has also been reported as produced by C. septicum.

Its toxicity is confirmed by subcutaneous injection of 1 ml. of the fresh culture of cooked meat broth into lab animals (guinea pigs) where it produces an extensive, spreading inflammatory oedema with slight to considerable gas formation in the tissues. It spreads by the bloodstream throughout the animal resulting in death in a day or two. Concentrations of the organism are found on the peritoneal surface of the liver showing long filamentous forms (type A only), and citron bodies, and in the heart where they multiply.

Autopsy shows extensive blood stained oedema with a deep red color, spreading from the site of inoculation over the abdomen. Muscles are turned deep red with a lot of gas but are not friable or softened.

<u>Protective Immunity, Resistance and Antibiotics</u>

Type A antitoxin provides protection against both type A and B infection toxins while type B only protects against its own set of toxins. Agglutinating antiserum is prepared in rabbits using both types of the organism as antigens.

<u>Incorporation into Weapons</u>

C. septicum is very pathogenic to most animals. Once in the tissues in anaerobic conditions it grows rapidly producing gas and a serous edema. They invade adjacent tissues and the bloodstream producing septicemia which is usually fatal in 24-48 hours. Its ability to invade and spread throughout the host target make it an effective biological agent when incorporated into anaerobic based ordnance. These properties make it an ideal add-on organism for the combination weapons already described.

Use of the organism in large numbers should easily overcome the commercial antitoxin serums because they do not yield high immunological content when derived from animals.

Clostridium Difficile

History and Recovery from Nature

First recovered and described in 1935 as a normal inhabitant of the intestinal tract of infants, it was not recognized until 1977 as producing a toxin and causing harm in man. It is present in about 64% of infants from 1-8 months in age in large numbers. It can cause fatal pseudomembranous colitis in humans when antibiotics are used. From 1943, it was often observed that when antibiotics were administered to lab animals, many died from enterocolitis. It was believed to be the result of overgrowth of gram negative bacteria, but in 1977, in Clindamycin induced colitis in hamsters, the organism and its toxins were finally isolated.

As a normal inhabitant of our intestinal tract, C. difficile is found in small numbers. It is recovered from only about 3% of the adult populations of the US and about 10% in Japan. When antibiotics are given orally, the normal intestinal flora are killed off. C. difficile, which is resistant to many antibiotics like clindamycin, cephalosporins, erythromycin, gentamycin, vancomycin and ampicillin, then grows to fill the void left in the intestines. In large numbers, it produces an endotoxin that is released on its death (lysis) causing diarrhea, and in some patients enterocolitis. Other clostridia species also flourish in the lower intestinal tract when antibiotics are used, but only the C. difficile usually produces toxin under these conditions.

Growth and Production

The bacillus is long, slender, and gram positive, about 6-8 x .5 um in size. They form subterminal spores which later become terminal and are smaller in diameter than the parent cell and begin to distend the parent body. They are motile with peritrichous flagella and colonies are grown on ordinary solid media with or without carbohydrates under strict anaerobic conditions. It hydrolyzes gelatin but does not digest casein. It ferments glucose but not maltose, lactose, or sucrose. It does not form indole or hydrogen sulphide.

C difficile grows at 25-45 C, but best at 30-37 C. It can be isolated on mediums with cycloserine and cefotoxin added. The antibiotics mentioned above also aid in its identification. Freshly isolated strains fluoresce under ultraviolet light. Colonies are 2-3mm in diameter after 48 hours incubation. They are slightly raised, white, opaque and circular with an entire margin. It does not produce hemolysis, is non proteolytic, and negative on egg yolk.

It is tolerant to cresol which it produces during growth. By adding .2% paracresol to the medium you can easily isolate it from other organisms.

Toxicity and Harm

The culture filtrates of C. difficile kill lab animals when injected by most routes. It causes cecitis in guinea pigs when introduced rectally. These effects are due to two separate toxins designated A and B. These toxins act together to cause the symptoms and injury observed in infections. They are produced by all strains of C. difficile.

Protective Measures

Immunization with toxoids from either toxin does not fully protect from the toxins. Both must be included in immunizations to be effective.

Incorporation into Weapons

The toxins will rapidly bind to the target cells in tissue cultures and at this point, antitoxin no longer works to neutralize its effects. This means that the development of vaccines, and antiserum would not work. Within a few hours of administration, cytopathic changes begin to occur in virtually all cell types.

The combination of rapid binding to cells which leaves the efforts to immunize or treat virtually worthless, and its ability to affect almost all types of cells makes this an interesting addition to anaerobic cocktail weapons. The addition of both the spores and the toxins will enhance this type of ordnance.

Clostridium Histolyticum

History and Recovery from Nature

C. histolyticum is widely, but sparsely distributed in the intestinal tract of humans and the soils around which they live and solid waste is distributed. It is associated with gas gangrene but is never implicated alone, and is recovered in 3-6% of all cases.

It surface colonies grow both aerobically and anaerobically which aids in its isolation and identification. The aerobic colonies are small and made up of mostly filamentous and pleomorphic forms. They grow much faster anaerobically and after 24-48 hours the surface colonies are roughly circular, about 1mm in diameter, opaque, gray-white in color with a shiny surface and entire edge. They do not tend to grow across the entire surface of the medium.

The aerobic colonies take about 48 hours to reach .5 mm in diameter. These are hemispherical, transparent, and do not grow further in size.

It ferments no sugars and is strongly proteolytic, attacking gelatin and more complex proteins. It is the only common Clostridia that produces gelatinase and is simultaneously glucose negative. It produces large volumes of hydrogen sulphide but not indole.

Heating and use of Neosporin in media inhibits most other organisms. By subculturing both aerobically and anaerobically, colonies can be identified for testing.

Growth and Production

The bacillus is long, slender, and gram positive, about 6-8 x .5 um in size. They produce large, oval subterminal spores that distend the organism. Filamentous forms develop in old cultures and aerobic conditions where sporulation does not occur.

Colonies grown on horse blood agar are surrounded by a narrow zone of hemolysis. On heated blood agar a large zone of proteolysis occurs with the development of wide zones of partial clearing around the colonies. When milk is added to the medium it is attacked. It does not react with egg yolk or ferment lactose. These combinations make identification nearly certain.

It grows well in cooked meat broth in 24 hours at 37 C or at room temperatures where vigorous proteolysis of the meat particles takes place.

Toxicity and Harm

There are many antigenically distinct, soluble toxins produced and recovered in filtrates.

Alpha toxin which is lethal and necrotizing and is antigenically related to the alpha toxin of the Cl. septicum already described.

Beta toxin which attack azocall and casein. It is a collagenase that destroys collagen fibers and markedly disrupts tissues in gas gangrene cases.

Delta toxin is a proteolytic enzyme called an elastase. It attacks azocoll and gelatin and is partially inhibited by reducing agents.

Epsilon toxin is a typical oxygen-labile hemolysin which is neutralized by antiserum from other related oxygen-labile hemolysins.

Gamma toxin is a proteinase activated by reducing agents. It does not attack native collagen but does attack azocoll, gelatin, and casein.

Other soluble toxins effects have also been observed including a peptidase, a gelatinase, and in one strain of C. histolyticum, at least nine (9) separate proteinases.

When a 1ml inoculation from 24 hour, fresh cooked meat broth culture is injected intramuscularly into the leg of the guinea pig, death occurs in 2-3 days. Edema first develops at the site of inoculation and spreads up over the abdomen. The inoculated muscles begin to break down and the skin ulcerates. Within 24 hours, the entire bone is denuded of soft tissues. The toxins digest the joint ligaments and the digestion of all the tissues may result in auto amputation at the hip joint.

Protective measures

Because of its huge range of toxins and its rarity in gas gangrene infections, no antitoxins or vaccines have been prepared.

Incorporation into Weapons

Its overall effects of drastically attacking all types of tissues and producing a huge range of toxins that cannot be immunologically protected against make this a strong candidate for offensive biological warfare ordnance. By attacking all tissues and producing horrific symptoms and injury, a strong psychological effect is induced on those who have to care for the recipients of C. histolyticum attacks. Immunological defense against anaerobic attack by this organism is non existent at this date.

C. histolyticum organisms and toxin make powerful additions to anaerobic cocktail ordnance.

Clostridium Sordellii

History and Recovery from Nature

C. sordellii is widely distributed in the soil. It is also commonly found in the intestinal flora of humans. Not all strains are pathogenic. It is recovered in about 4% of all gas gangrene cases.

C sordelli is urease positive and does not ferment mannose, sorbitol, and salacin. Colonies on horse blood agar (3%) have crenated or coarsely rhizoidal margins. It sporulates freely in culture with the spores large and cylindrical. They slightly distend the cell body and are centrally placed. The colonies are usually surrounded by a narrow zone of hemolysis. On heated blood agar, a partial clearing is produced from proteolysis. On egg yolk and milk, extensive opalescence is produced. The milk is attacked with zones of partial clearing around colony growth.

At 24 hour incubation the surface colonies are 2-3mm in diameter, are gray-white, with a low convex surface and an entire or irregular edge. It is common for swarming growth to occur. In broth cultures, a viscous mucoid deposit is produced which is peculiar to this organism.

Spores are heat resistant and broth's may be cooked to eliminate most other organisms with Neosporin added to yield mostly Clostridia species during isolation.

Growth and Production

The bacillus is strongly gram positive measuring 2-4 x 1 *u*m in size. Free spores are common and chains of sporulating organisms are often seen under the microscope. In fluid mediums like broth, chain and filament formation is common which helps in identification and separation.

Good growth is obtained in cooked meat broth in 24 hours and the meat particles are partially digested. It ferments glucose and maltose but not lactose or sucrose. Gelatin and complex proteins are broken down. Indole and hydrogen sulphide are produced.

Toxicity and Harm

All strains produce a lecithinase C that is antigenically related but not identical to the lecithinase C of C. perfringens. It causes lysis of red blood cells with the mouse very susceptible and sheep and horse resistant. One other soluble toxin has also been observed in filtrates.

Intramuscular inoculation of the organism (1 ml) into guinea pigs produces death in 1-2 days. On autopsy there is a subcutaneous gelatinous oedema spreading along the surface away from the inoculation. The muscles hemorrhage with some gas formation present. The proteolysis proceeds like C. histolyticum where the bones of the inoculated limb become denuded of soft tissues and self amputate at the hip joint.

Protective Measures

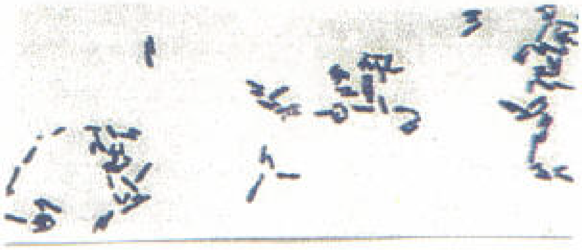
The lecithinase C is related to C. perfringens and is partially inhibited by its antitoxin.

Incorporation into Weapons

Besides the lecithinase toxin, another lethal toxin is also produced. In gas gangrene with this organism, large amounts of edema similar to that of C. novyi is produced.

This is one more organism that can be added to the anaerobic weapons cocktail. The incorporation of many organisms and toxins in combination makes it nearly impossible for preventative measures involving immunity to be effective. Likewise for antitoxin. The target populations are ultimately, medically helpless against these types of combination attacks.

The diptheroidal appearance of C. diptheria under the microscope showing the "chinese letter" or "picket fence" arrangement of the bacteria clusters.





C. diptheria gravis with almost no granules. Grown on Loefflers serum, the cells are short and stain evenly.

C. diptheria intermedius have few granules. Cells are pleomorphic with some long, others short and many are club shaped.

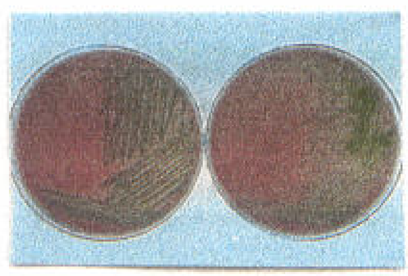




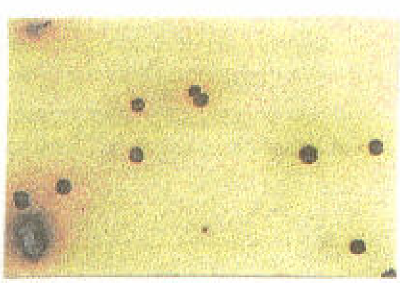
C. diptheria mitis with many many granules. The bacilli are long, curved and thin. They stain pale grey-green with the granules bluish-black.

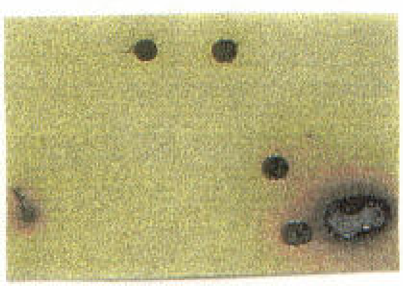
Tiny colonies shown on blood agar plates. This strain is showing an alpha hemolysin.

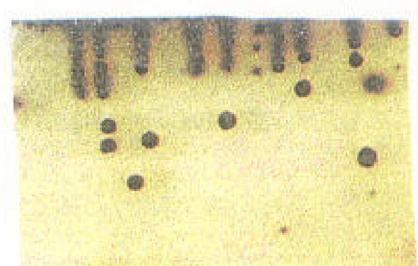




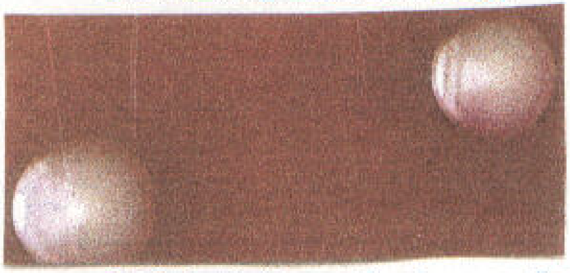
Agar growing C. diptheria without blood but with potassium tellurite added. Those colonies surrounded by the brown halo are the C. diptheria while those with discreet edges are Staphylococcus aureus.



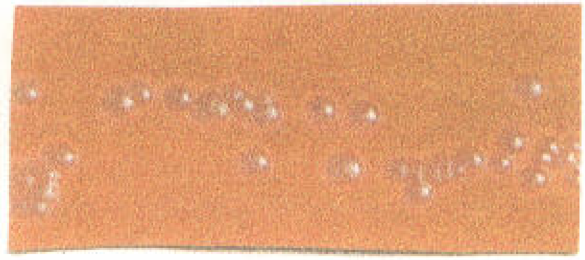




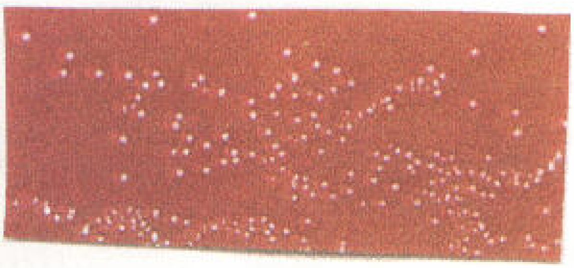
C. diptheria gravis grown on blood agar 18 hours at 37 C. Large with a dull flat surface faint radial striations at the edge.



C. diptheria mitis colonies with smooth surface and surrounded by a very narrow zone of hemolysis.



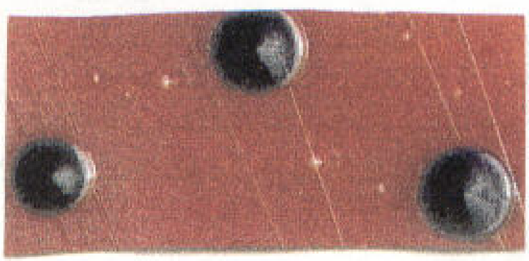
C. diptheria intermedius have smaller smaller colonies and are non hemolytic.



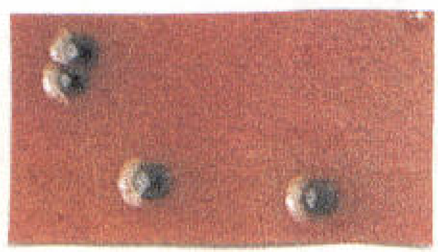
Gravis strain grown with tellurite added. Metallic gray centers and radially striated edges called "daisy head".



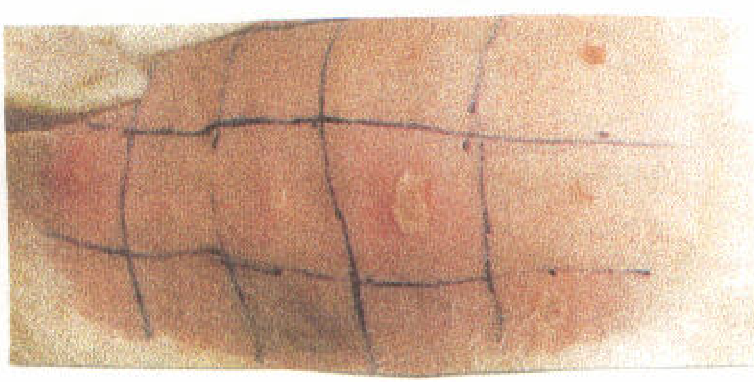
Mitis strain with a black raised center sloping to a pale rim "coolie hat".



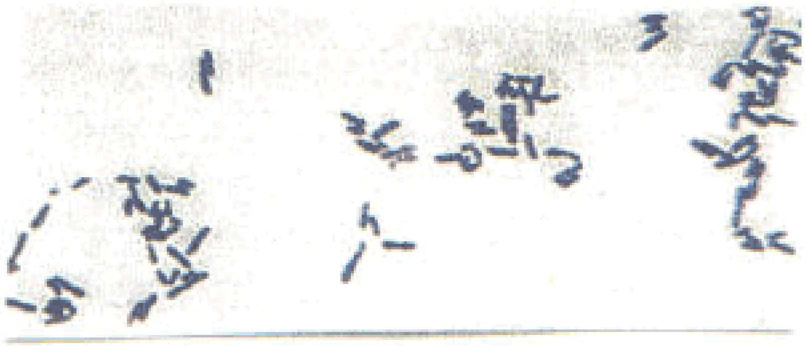
Intermedius strain with dark centers, small and light colored edges "frogs eggs".

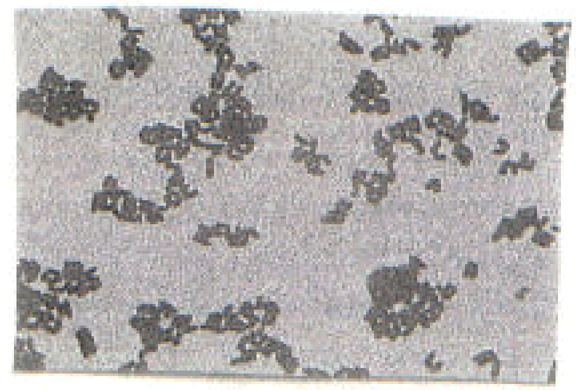


Intradermal injection test for toxicity in a rabbit. Notice the white zone of necrosis surrounded by erythema. Results are similar in guinea pigs.



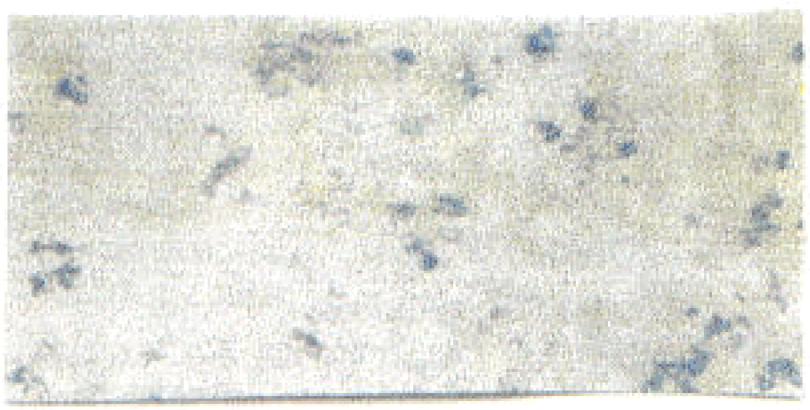
The diptheroidal appearance of C. diptheria under the microscope showing the "chinese letter" or "picket fence" arrangement of the bacteria clusters.

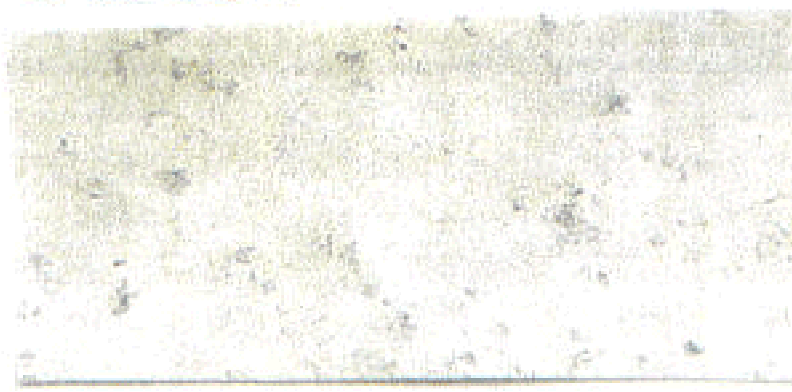




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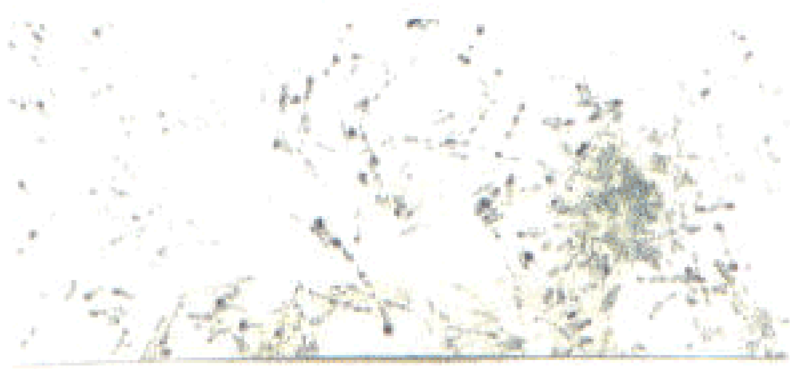
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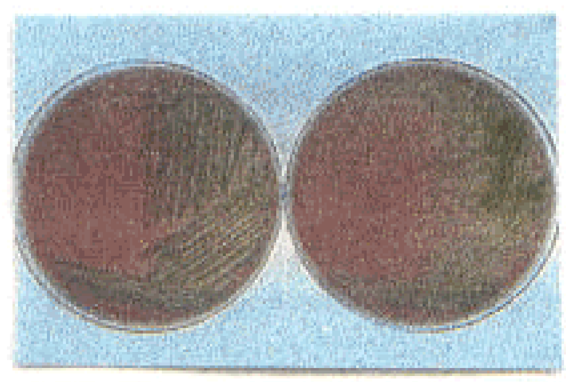




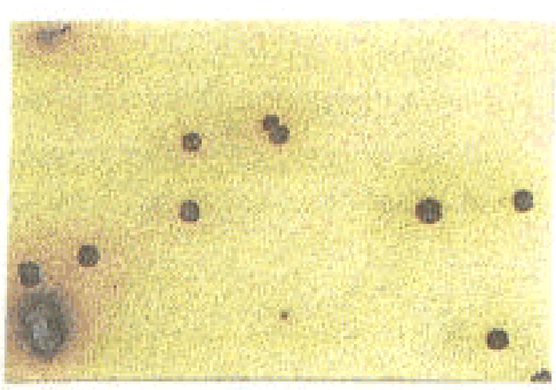
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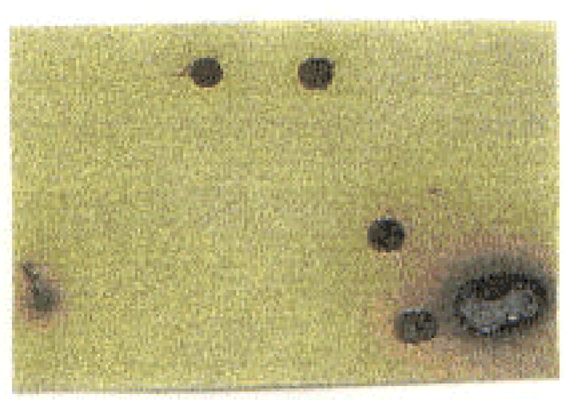
Tiny colonies shown on blood agar plates. This strain is showing an alpha hemolysin.

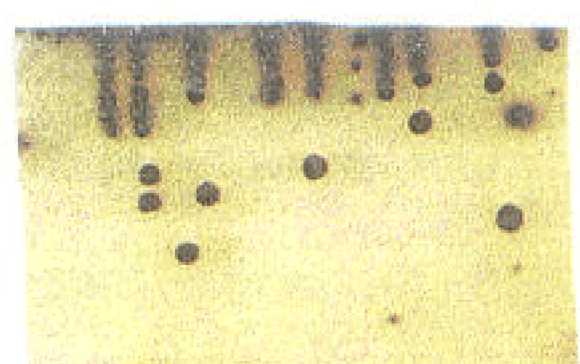




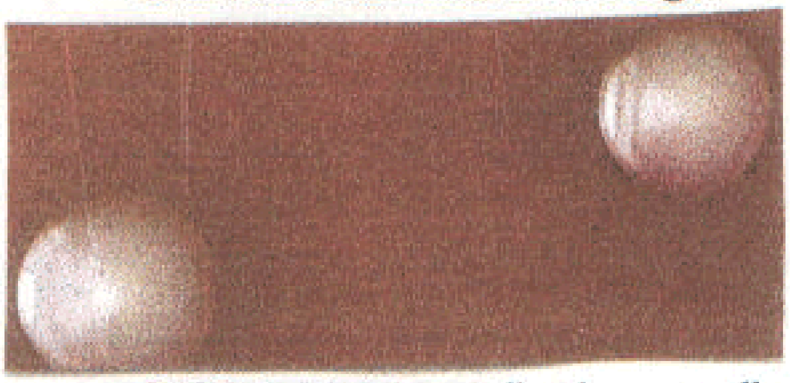
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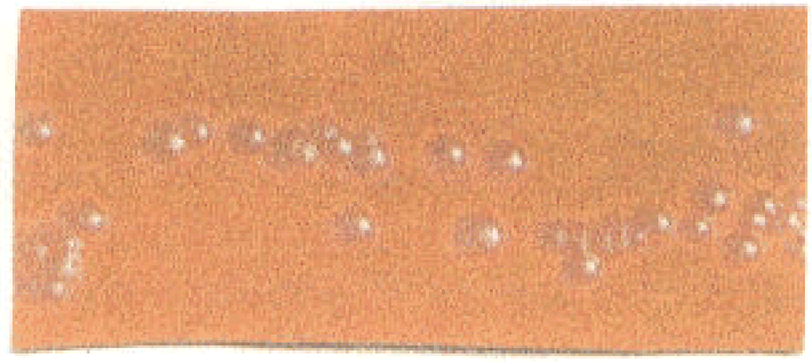




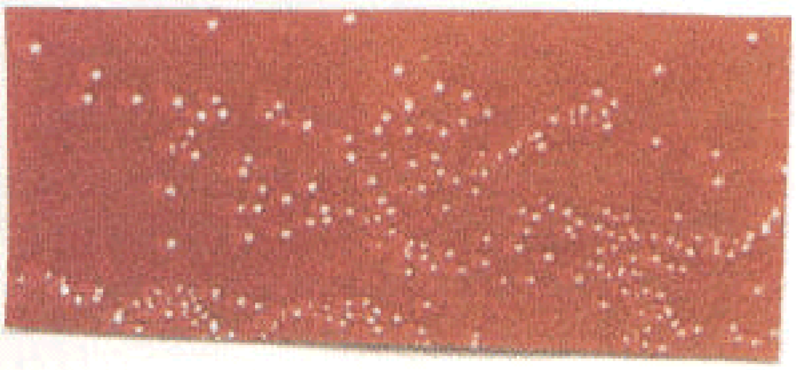
C. diptheria gravis grown on blood agar 18 hours at 37 C. Large with a dull flat surface faint radial striations at the edge.



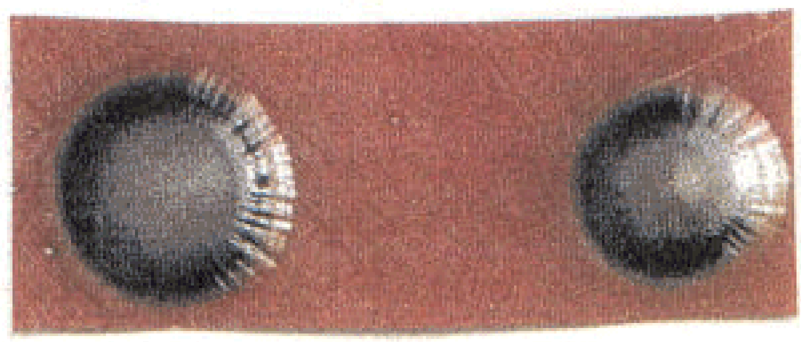
C. diptheria mitis colonies with smooth surface and surrounded by a very narrow zone of hemolysis.



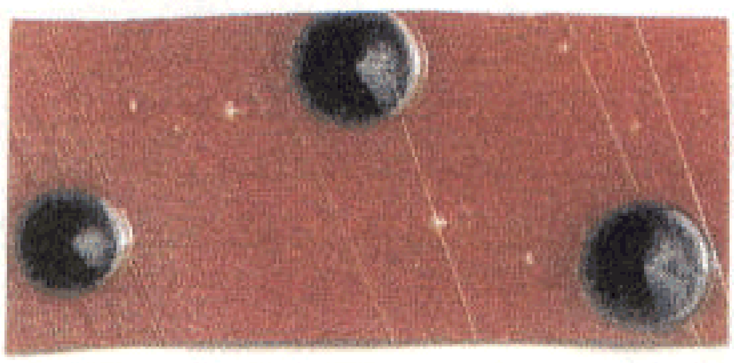
C. diptheria intermedius have smaller smaller colonies and are non hemolytic.



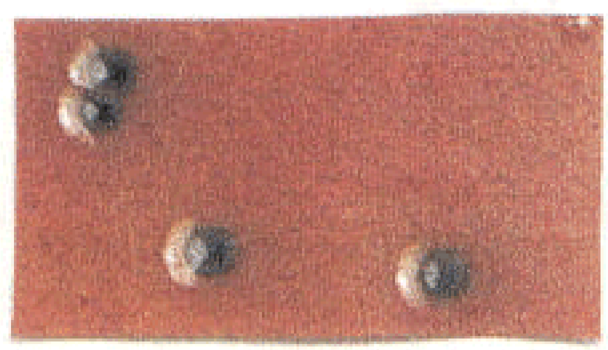
Gravis strain grown with tellurite added. Metallic gray centers and radially striated edges called "daisy head".



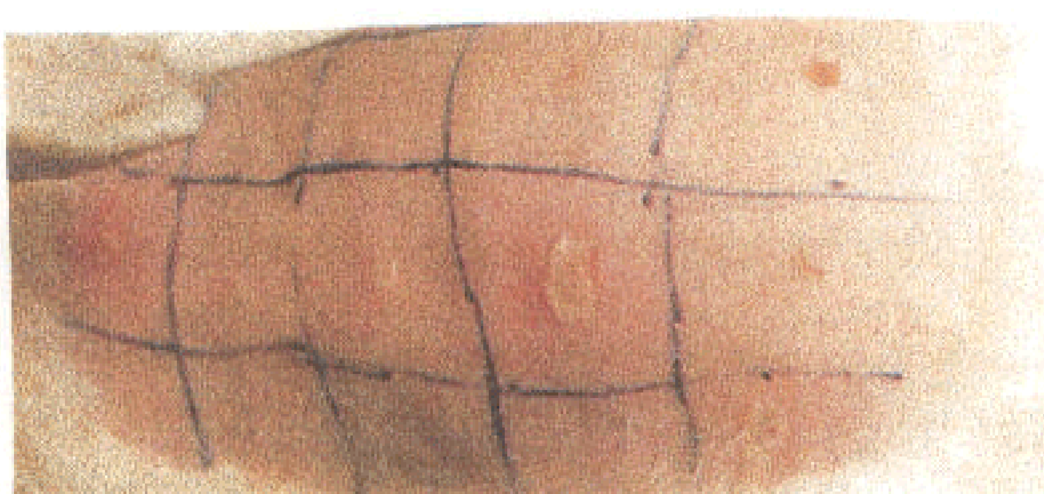
Mitis strain with a black raised center sloping to a pale rim "coolie hat".



Intermedius strain with dark centers, small and light colored edges "frogs eggs".



Intradermal injection test for toxicity in a rabbit. Notice the white zone of necrosis surrounded by erythema. Results are similar in guinea pigs.



Corynebacterium Diphtheria

History and Recovery from Nature

Corynebacteria are widely distributed in nature and commonly found in soil, water, the skin and mucous membranes of man and animals. C. diphtheria, the main infective member of this genus usually infects the upper respiratory tract or wounds. It is also found in or on healthy carriers.

C. diphtheria was first described by Klebs in 1883 who recovered it from the pseudomembranes of patients with diphtheria. Pure cultures were finally isolated in 1884 by Loeffler who developed a special medium on which to grow it. The mode of action was described in 1888 by Roux and Yersin who related the bacteria and its toxin to the disease. They isolated its toxin by calcium chloride precipitation from old alkaline cultures of the bacillus and found that 1/2mg of this precipitate killed guinea pigs.

Diphtheria is an acute, contagious illness characterized by local inflammation and formation of a pseudomembrane in the oropharynx. The causative bacteria, C. diphtheria produces a potent exotoxin that damages the heart and peripheral nerves. It has historically been a health problem in the southern US where it is both endemic and at times epidemic. Immunization has reduced its incidence in this country from about 2,000 per million people in 1920 to less than 1 per million by 1976.

It has been primarily a disease of early childhood with peak incidence in children of 4-6 years of age. Infants are usually protected by immunity passed on from their mothers and lose this in 3-4 years in the absence of immunization. The infection is spread by close contact at schools and other public institutions. Spread by accumulations of the bacillus on dust and fomites seem greater than that by direct droplets that are sneezed or coughed. It also occurs as, and can be spread from skin ulcers. This is especially prevalent in the tropics where hygienic conditions are poor and the organism can be recovered from open sores. Cutaneous diphtheria is common in these tropical areas providing a reservoir of infective organisms and are called desert sore or tropical ulcer.

In the skin infection, the diphtheria degenerates the epithelial cells extending into the underlying tissue yielding a profuse fibrinous exudation. The bacillus rarely invades beyond this local lesion but still produces toxin.

C. diphtheria is spread by convalescent and healthy carriers in their respiratory tracts. An incubation period of one day to one week follows exposure by a susceptible individual. During infection it grows in the nasopharynx and elsewhere producing an exotoxin that causes necrosis and a superficial inflammation of the mucosa.

The bodies response to the toxin and necrosis of the area is to produce a large amount of fibrinous exudate. A grayish to black pseudomembrane forms from exudates of dead cells built on this framework of fibrin. It adheres to the throat tissues and if attempts are made to remove it forcibly, a raw, bleeding surface is exposed.

C. diphtheria does not invade the submucosal tissues. Its exotoxin is produced usually in the throat and diffuses into the surrounding tissues and circulatory system where it is carried to the major organs. The major sites it attacks are the heart and peripheral nervous system as well as affecting other tissues as the disease progresses. Diphtheria has a mortality rate of 10-30% depending on various factors such as obstruction of the airway due to pseudomembrane growth in the larynx or trachea, and congestive heart failure. If the airway is not restored by the use of a tube or tracheotomy, the patient usually suffocates. Heart failure is due to fatty myocardial degeneration which leads to a collapse of the circulatory system. This damage is reversible if the patient survives.

The organism is recovered from throat secretions, swabs, or the removed pseudomembrane of affected individuals. There have been periodic outbreaks in the US, mostly in crowded slum areas with limited access to health care facilities. Crowding and close interpersonal contact during the winter months result in spread of the infection, especially in the south.

In order for some bacteria to produce a toxin, they must first be infected with a virus called a "bacteriophage". Diphtheria is one of those bacteria. Only strains that have been infected by a specific bacteriaphage called beta phage can produce the toxin and cause the illness. It was first observed in the laboratory in 1951 and was remarkable to recognize that more than one microorganism was required to produce this disease. This is why people can carry the C diphtheria around for years without illness. The beta phage carries the structural gene necessary for the C. diphtheria to produce the toxin. Nontoxic strains of the organism are often isolated from carriers during outbreaks.

In order to tell if the strain you have isolated is truly toxin producing, it is tested in animals. To differentiate from other bacteria, the C. diphtheria is a gram positive bacillus which easily decolorizes, and it does not produce endospores or capsules. Near the end of cell division, there is a "snapping" where the bacilli remains attached at sharp angles to each other which gives it an easily identifiable appearance of Chinese letters under the microscope. It grows both aerobic and anaerobically but growth is sparse without air. A pH of 7.8 -8 is best.

C. diphtheria does not produce hemolysis, is not motile, does not produce hydrogen sulphide or attack esculin or salacin. It ferments glucose and mannitol.

Loeffler developed a special medium for growing C. diphtheria consisting of -

3 parts beef blood serum
1 part of 1% dextrose

The pH is adjusted to 7.2 and is usually dispensed into test tubes which are slanted and then heated for 4 hours at 85 C. The oven is turned off and the tubes are left overnight. The next day they are autoclaved at 121 C for 10 minutes to insure the outside is sterile.

Loefflers blood serum allows luxuriant growth of C, diphtheria yielding minute, glistening, gray-white colonies in about 18 hours at 37 C. The colonies grow at 15-40 C but best at 34-37 C. This media is considered to be a starvation media which aids in growing some bizarre forms which aids in identification. This medium also inhibits most other related infectious organisms like streptococci and pneumococci. It is also deficient in some nutrients that C. diphtheria needs and the result is unbalanced cell wall synthesis. This results in the pleomorphic, easily recognized colony morphology described above.

This organism also grows well on many ordinary media (with contaminants) and its growth is improved with the addition of tellurite salts (potassium tellurite) and cystine. The tellurite also inhibits growth of many other microorganisms. The various species of Corynebacteria reduce the tellurite to different degrees allowing for easier identification. The reduction of tellurite produces a darkening of the colonies with the diphtheria strains allowing for differentiation -

C. diptheria-gravis	Battleship gray to black, flat, dull, dry with radial striations, called "daisy head" formations, friable and colonies may be 4mm in diameter. Growth in broth is slightly granular, soft pellicle.
C. diptheria-mitis	Black, smooth, convex, shiny, and butyrous, rarely exceed 2mm in diameter. Growth in broth yields a finely granular uniform turbidity.
C. diptheria-intermedius	Dark gray with an even darker center, umbonate, friable, small, only 1mm in diameter. Irregular margin like a poached egg. In broth it can be granular, flakes, pellicle, variable.
Diptheroids	Grows in greater abundance than above and may have any of above characteristics.

In culture, the diphtheria bacilli can survive for two or more months at room temperature. When dry they survive much longer. The bacilli may remain virulent and alive in the dust of homes of infected individuals for many months after infection.

Diphtheria bacilli have survived on dried pseudomembranes for up to 14 weeks. Boiling will kill them in two minutes and they die at 60 C in 10 minutes. They are resistant to drying, freezing, and sunlight. Ultraviolet light assists in producing toxic mutations with bacteriophage.

Most disinfectants kill C diphtheria and they are sensitive to penicillin and tetracyclines. Continued culture on laboratory mediums usually reduces the virulence of the strain.

For the serious weapons producers, the surface K antigen, which is a heat-labile protein located in the superficial layers of the cell wall assists in invasiveness of the organism. An additional cord factor described in the literature combines with the K antigen to increase virulence of a particular strain. It has also been found that the addition of a neuraminidase enzyme assists in the ability of the organism to invade the throat tissues. This enzyme acts in the mucus to provide a readily available source of energy for the bacteria invading the mucus membrane.

Man is the only animal host of the bacteria and the use of widespread immunization has nearly eliminated the disease incidence in North America.

Growth and Production

Under the microscope the organisms appear as slender, straight, or slightly curved rods measuring 1.2-6.4 x .3-1.1 um. They rarely are uniform in thickness and often have club shaped thickenings at one or both ends. A few may be thickest in the center and taper off at the ends. Internal structures can be seen when grown on Loefflers medium. Some forms may appear pear shaped, club shaped, or globular. When stained with methylene blue it shows a beaded or barred appearance.

It grows on all ordinary nutrient media but best on serum media. When grown on Loefflers serum the colonies at first appear small, circular, white opaque discs that later become thicker and the borders crenated. Sometimes the growth shows a distinct yellow tint.

In broth, some strains grow in small white masses which precipitate as sediment in the tube or sticks to the sides. Surface film of growth also develops. Other strains will produce a uniform growth throughout the broth. By adding potassium tellurite, the bacillus reduces the tellurite which yields grayish to black colonies.

The gravis and mitis forms of diphtheria described earlier are the severe (gravis) and mild (mitis) forms of the disease although the intermedius may be deadly. The is related more to the ability to invade the tissues rather than production of toxin.

C. diphtheria ferments glucose, galactose, maltose, and dextrin with acid production that liquefies gelatin. It does not ferment lactose, sucrose, or mannitol. There is no proteolytic activity.

Gravis strains ferment starch and glycogen and this is observed by adding .1% peptone water in serum medium. Acid production causes clotting in the medium. Mitis strains hemolyze ox or rabbit blood, the gravis lyses rabbit blood only, and the intermedius is non hemolytic.

Toxicity and Harm

The toxin that is produced by the bacillus at the infection site diffuses rapidly into the adjacent tissues and bloodstream. The toxin is quickly absorbed into the mucous membranes of the throat and it rapidly destroys the epithelium. This necrotic epithelium becomes imbedded in exuding fibrin, erythrocytes, and leukocytes forming the grayish "pseudomembrane" that begins to cover the tonsils, pharynx, and larynx. If the membrane is not removed or a tube placed down the throat, the individual may suffocate. Continued toxin production results in accumulations in the circulatory system and carried to the heart muscles, nerve cells, kidneys, liver, and adrenal glands for which the toxin has a special affinity. Very tiny amounts of toxin can produce extensive damage to all these tissues.

The bacilli are present in large numbers in the exudate, throat secretions, and pseudomembrane. They do not invade the blood or lymphatic system. In nasal diphtheria characterized by a crust formation, the bacilli are present in blood stained nasal discharge.

The diphtheria toxin is actually two distinct substances that are produced together by all strains and are designated fragments A and B. The different ratios of A and B produced by the strains account for the difference in the toxicity. The A fragment is the actual toxic component but it requires B fragment to aid in its spread into the tissues. In the absence of B the strain is not considered very deadly. With large amounts of B produced, the strain is rendered "hypertoxic". This is because the B fragment interacts with surface receptors on the cell walls that allow it to open up and permit the entry of the entire toxin molecule. The fragments are produced and travel together as part of the same molecular complex. The toxin is considered hemorrhagic, necrotic, and lethal. It destroys cells by the enzymatic inhibition of protein synthesis.

When infective diphtheria bacilli are grown in suitable fluid media, abundant toxin is produced. Human serum aids in the production of toxin. Artificial mediums used to grow the diphtheria are published in the various microbiology texts and usually include the necessary amino acids, inorganic salts, maltose, and growth factors - pimelic acid, nicotinic acid, and *B*-alanine.

Toxin production in diphtheria requires a specific range of iron in the medium. This is app. .14 to .5 micrograms per ml. which explains why it does not produce toxin when living off of most human tissues. The production of toxin peaks as the iron becomes deficient in the medium. This lack of iron apparently causes the cells to produce and release toxin into the surrounding medium. As much as 5% toxin can be produced (as a % of the bacterial weight) in starved media.

Toxin produced and released by the cells is non toxic until acted upon by proteolytic enzymes. The addition of trypsin activates the toxin. This toxin then is capable of inhibiting protein synthesis in the cells of animals exposed to it. This toxin is lethal to humans, rabbits, guinea pigs, and birds at doses of 160 ng/kg body weight. A single invisible particle of dust can carry many lethal doses by itself.

The toxic liquid is filtered off and concentrated by fractionation with potassium phosphate or a combination of sodium carbonate and ammonium sulfate to avoid co-precipitation. The purified toxin yields an LD50 of .0001 mg for a 250 gram guinea pig.

Maximum toxin production is achieved in a pH of 7.8-8 (acid pH inhibits toxin formation), under aerobic conditions for 7-10 days at 37 C. The toxin is unstable at pH of 6.0 or less and is heat-labile.

The toxin is unstable and its potency diminishes on exposure to air and light. In sealed tubes and in the dark, it is stable for several weeks. It converts spontaneously to toxoid.

Permanent paralysis and death are the common result in untreated individuals.

Two methods of testing the recovered C. diphtheria cultures for toxigenicity are -

1. Guinea pigs are inoculated with a pure culture of C. diphtheria colonies. The virulent bacilli will produce a well defined area of inflammation surrounding the inoculation site within 24-48 hours. It fades in 3-4 days resulting in a minor necrotic patch on the skin or scab, surrounded by growing hair. If the dose is large, the animal will die in 1-4 days. Autopsy reveals edema and possibly necrosis at the site of injection, congestion of the regional lymphatics, and abdominal viscera. Enlarged and hemorrhaging adrenals and a pleural exudate are also seen in this animal.

A control animal is injected with antitoxin (if available) and will not show inflammation. Rabbits may also be used for this test procedure.

2. Strips of filter paper are soaked in diphtheria antitoxin. This is placed onto the medium. The antitoxin diffuses into the medium away from the strip and when the culture produces toxin corresponding to the antitoxin, the two meet in optimal proportions and form a thin line of precipitation. Many cultures can be tested simultaneously by this method.

Those cultures that do not produce positive responses should be considered non-pathogenic.

Protective Measures

Diphtheria antitoxin is the only effective treatment. It must be administered early, before the toxin irreversibly binds to target cells. Fatality rates are nearly zero when antitoxin is given the first day of the disease. It reaches 5% by day two and 20% by day five as more toxin is produced and more organs and tissues are affected. This is because the toxin binds irreversibly to the cells it attacks. The antitoxin binds to fragment B preventing the entire toxin from attaching to the human cells. It is necessary for the antitoxin to be applied in large amounts to bind to circulating toxin, and quick enough to reach the toxin before it has already attached to and damaged the target cells.

Antibiotics such as penicillin, tetracycline, and erythromycin will kill the bacteria quickly but offers no protection against the toxin.

Active immunization with toxoids offers good protection in ordinary infections. Passive immunization with antitoxin is used for prophylaxis and treatment. The antitoxin depends on its ability to neutralize the B fragment to prevent the spread of the A component. The A fragment has almost no antigenic components on its surface to use in toxoid. This means that the antitoxin must be produced from a strain that is rich in B fragment material. Antibodies directed against fragment B material neutralize the toxin very effectively. For large scale toxin production used commercially, the single strain "Park-Williams #8" has been universally used.

Toxoid is prepared by adding 3% formalin to toxin and incubating it for 2-3 weeks at 37 C. It is tested by injecting into guinea pigs. If there is no reaction, a complete change to toxoid has taken place.

It can also be produced by the addition of potassium alum which precipitates it and when resuspended has increased antigenic ability because the alum toxoid remains in the subcutaneous tissue for a long period providing antigenic stimulus. The toxoid is usually redissolved in sodium citrate or sodium tartrate.

Immunization against the diphtheria bacillus itself is difficult because of its location in its infective period where antibodies cannot easily reach it and the fact that more than thirteen types of gravis strains have been classified by agglutination reactions with antisera. Four types of mitis and more than forty types of intermedius have been identified. Immunization has also not stopped the disease but it has significantly lowered the fatality rates.

In 1913, Shick introduced a method for detecting resistance or susceptibility to the toxin. He injected a small amount of toxin under the skin which produced a red induration in 24-36 hours that lasts four days or longer in susceptible individuals. No reaction indicates they are immune under ordinary circumstances. A control of toxin that has been heated at 60 C for 15 minutes is used in the other arm to identify other skin reactions.

Escherichia coli

History and Recovery from Nature

Escherichia coli (E. coli) were discovered in 1886 by Escherich and are a normal inhabitant of the human and most warm and cold blooded animal intestines. It serves a beneficial purpose in suppressing the growth of certain proteolytic organisms and synthesizes a large numbers of vitamins which we use.

Coliform organisms are the measure used in determining in their is fecal contamination in foods, water, and other materials or fomites that humans come in contact with. The definition of "coliform" can mean just E. coli, or all enterobacteriaceae depending on who is using the term and how they intend it.

The E. coli is a widely used tool for microbiologists in genetic engineering and basic physiology. They produce a number of toxins which causes disease. They are the primary cause of pyogenic infections of the urinary tract, often alone or with fecal streptococci and also occur in appendix abscess, peritonitis, cholecystitis, septic wounds, and bed sores.

In the human intestine they can be found in large numbers as well as large numbers of strains. The strain types vary over time with some being transient and others persistent colonizers. Strains that do not cause disease in the intestine are often found in other locations as the primary pathogen, particularly in urinary tract infections (pyelitis and cystitis) of which they are the primary pathogen in 85% of the cases..

E. coli is responsible for many intestinal infections in livestock but those strains that cause intestinal disease in animals do not cause the same disease in humans and vice versa.

E. coli is aerobic and facultatively anaerobic. They grow well on all media producing large, moist, convex to domed, entire, opaque, butyrous colonies on blood agar in 24 hours. On gelatin they are translucent around the colony periphery and this makes them resemble maple leafs. Best growth is between 20-40 C and 6-8 pH. Optimum growth occurs at 37 C.

They are inhibited by brilliant green and by the combination of sodium desoxycholate and sodium citrate (SS and DCA agar) and do not grow well on these media. Both smooth and rough colonies may be produced and some strains grow well on lactose but may take up to seven days to show fermentation. On EMB media they produce a metallic sheen as already illustrated.

Most strains are encapsulated and motile using peritrichous flagella. Some strains are *A* or *B*-hemolytic on blood agar and these are more often the pathogenic strains.

Identification is made by fermentation of glucose, lactose, maltose and other carbohydrates with production of both acid and gas. They are indole positive, citrate negative, Voges-Proskauer negative, and methyl red positive. E. coli does <u>not</u> liquefy gelatin.

The indole test is important because another species (Aerobacter aerogenes) is widespread in nature and closely resembles E. coli. They can be told apart because the E. coli will degrade the amino acid tryptophan into indole while most other species do not. Testing for Indole is accomplished by adding 1% tryptophan to the growth medium and then incubating for 24 hours. At the end of the growth, 5 drops of the following "Kovacs" reagent is added -

Kovacs Reagent	
Pure amyl or isoamyl alcohol	150 ml
p-Dimethylaminobenzaldehyde	10 g
Concentrated HCl acid	40 ml

In seconds, the media will turn a bright red indicating the presence of indole showing that the test is positive and the organism is most likely E. coli.

E. coli survive in culture at room temperature for several weeks as can most other enterobacteriaceae. They can be maintained on egg saline for up to a year. Most disinfectants kill E. coli as will heating at 60 C for 20 minutes in broth media.

E. coli is the most frequent cause of urinary tract infections and often causes appendicitis, peritonitis, postoperative wound infection, and infant diarrhea. Epidemiological studies indicate that most E. coli infections (45%+) are acquired in hospitals making this one of the best sources for the most virulent strains. The stool, pus and urine specimens produce the most concentrated sources but these always contain large numbers of indigenous strains.

Disease producing E. coli strains are easily and often recovered by tourists visiting developing countries with untreated water supplies. It is also the leading cause of hospital acquired infections and pathogenic strains are easily recovered there as well. Most strains produce diarrhea or dysentery which continues until exposure produces a built up immunity and the disease stops.

Growth and Production

E. coli are short, plump rods .4 *u*m x .7 *u*m wide and 1-4 *u*m long. They uniformly stain gram negative and capsules may be observed in some strains. Their motility ranges from sluggish to non-motile. Fresh broth cultures sometimes produce coccoid forms that yield short chains.

Almost any simple medium permits the large scale mass production of E. coli making it easy to rapidly mass produce for use as a weapon. They produce large amounts of lactic acid and smaller amounts of formic and acetic acids during production which significantly lowers the pH of the surrounding medium.

Toxicity and Harm

The antigenic makeup of E. coli is complex but most members possess three distinct types or groups of antigens. Members of all (most?) of the family of Enterobacteriaceae contain these as part of the mosaic of their antigens.

a) "O" or "somatic" antigens which are closely bound to the E. coli located within the cell or near its surface and are heat stable for 30 minutes at 100 C. The name comes from the German "ohne hauch" or non spreading. These antigens are polysaccharides. There are over 160 "O" groups that have been identified.

b) "K" or "capsule, envelope" antigens which occur mainly in E. coli that produce capsules. These antigens can be destroyed by boiling the organisms for 30-60 minutes. These antigens may cover up or mask the O antigens and it is believed that by boiling the E. coli, the K antigens are removed leaving the O antigens more accessible to antibody in identification tests. From the German "kapsel" or envelope, these antigens surround the cell. Not all E. coli have K antigens and one type is found in a given strain. Strains with the K1 antigens have been implicated in neonatal meningitis. The K antigens with fimbrial structures can be transferred from one E. coli to another by plasmids while the other types of K antigen are controlled by the genes.

c) "H" or "flagellar" antigens which are also thermo-labile (destroyed by heat) at 100 C for 30 minutes. From the German "hauch" or spreading due to the fact that most bacteria with these antigens are motile. These antigens are located on the flagella and are proteins. There are more than 50 H antigens known.

Some species contain a "Vi" antigen that is believed related to virulence of the organism and is a surface component. These often mask the true antigens making them inagglutinable. This phenomenon can be made inactive by boiling for 30 minutes because they are heat-labile.

E. coli are serotyped on the basis of their O and K antigens.

Three types of K antigens have been described and are labeled L, A, and B. Most of the pathogenic and deadliest toxin producers have at least one K antigen of the B variety. This is because the K antigen capsules allows the E. coli to resist phagocytosis and the bactericidal effects of human serum.

In order to test and identify E. coli serologically a slide agglutination test is done by -

a) taking a colony from agar or broth and add to saline in a test tube. This forms a milky suspension.

b) On a separate spot on the slide (mark with a grease pencil) add a small drop of antiserum.

[Antiserum are commercially available from Difco laboratories, Detroit, Michigan, Baltimore Biological Lab's, Cockeysville, Maryland, and through most medical supply companies.]

c) Add a second drop of culture to the antiserum and mix it by gently rocking the slide.

When the antigens of the cells match the antibodies in the antiserum, agglutination should occur immediately with the formation of fine granules and/or large aggregates. If the E. coli fails to agglutinate any of the O antisera, heat the culture to 100 C for 30 minutes, cool, and then repeat the steps. By heating you are removing any masking K (capsular) or Vi antigens that may have interfered with the test.

Members of E. coli are all alike biochemically but can be divided into several hundred groups according to their O antigens and can then be further typed by the presence of K and H antigens. In the following tables, the main serotypes associated with disease are listed. The first number follows the "O" identifying which somatic "O" antigen it possesses. The second number represents the capsular or K antigens which are further grouped as B-type capsular antigens. The three columns of serotypes (Poly A,B, and C) represents groups of serotypes for which polyvalent antisera have been pooled and made commercially available. By first testing with the polyvalent antisera you can tell which group the colony belongs to and then need only test those in that group.

Poly A	Poly B	Poly C
O26 :B6	O86 :B7	O18 :B21
O55 :B5	O119 :B14	O20: B7
O111 :B4	O124 :B17	O20 :B84
O127 :B8	O125 :B15	O28 :B18
	O126 :B16	O44 :K74
	O128 B12	O112 :B11

[The above list is an older one. The newer strain O157 :H7 has been in the news in recent years. It is found in about 60% of all the cattle herds in the state of Nebraska and was responsible for the closing of the Hudson foods plant in Columbus, Nebraska in 1997. Although the source of the E. coli was never determined, the author believes it originated from a nearby dairy herd. One of the contractors had been working on the farm during which a ladder became covered in manure. This dried on the equipment and a week later the company was called to work at the site where the ladders involved were carried into the plant interior without being washed. The dried manure may have contained millions of cells per gram of the suspected strain.

This author received various inquiries concerning the fact that I write about biological weapons and the incident occurred 20 miles from my home as well as the fact that the source of the strain was never identified. It was also implied to me that US army bio weapons experts were among those USDA investigators. I have no way of knowing as of the date of this publication as to whether this is true or not and given the US governments track record of not telling the truth, it is unlikely if the public would ever be told anything by this government that it could actually have confidence in anyway.

This same strain is also responsible for the June 1998 outbreak in Georgia that sickened 4,500 people. Part of the spread occurred in a pool in which one of the children (with diarrhea) is believed to have defecated in the pool distributing the organism and infecting others present at the time. It is clear that this strain is easily obtainable, can be easily mass produced and distributed, and can cause widespread harm. It was responsible for consigning a billion dollar company to the trash heap of history in little more than two weeks attesting to its potential as an effective military scale weapon.]

Another substance is produced by E. coli that is bactericidal for other microorganisms. This protein is called **colicin**. The colicin of one strain of E. coli may kill not only other species, but other strains of E. coli as well. The colicins bind to specific receptors on the outer membrane of susceptible cells and then they act to penetrate the cytoplasmic membrane or form an ion permeable channel in the membrane after which the membrane collapses. Other colicins may also interrupt protein synthesis by various means. Colicin production allows one strain of E. coli to crowd out other bacteria and non pathogenic strains of E. coli improving its colonizing surface area.

Pathogenic E. coli produces one or more types of **Enterotoxins** that mediate the movement of water and ions from tissues into the bowel lumen. This yields a net secretion that we call diarrhea. The two different kinds of enterotoxins are ST (heat-stable) and LT (heat-labile). They both bind to receptors on cells and then causes the cell to produce a substance that causes water and electrolyte loss in the tissues. This mode of action (the LT toxin) is very similar to the cholera toxin to which it is immunologically related. The potency of the LT toxin is only about 1% that of the cholera toxin possibly due to different binding sites in the small intestine. The ST toxin also induces water and electrolyte secretion in the intestine.

In rabbit tests the ST toxin produces maximum effect in about 4 hours while the LT toxin takes about 10 hours for peak effect. The test for toxin production in animals is done by injecting the suspect colony into the eye of a guinea pig. Only the invasive strains are able to colonize the eye tissues.

Some E. coli's also produce a hemolysin and when these are recovered they are likely disease producers which make the best weapons. The *A* hemolysin is a soluble protein that is released from the cell. Its production is controlled by transmissible plasmids meaning it can be acquired by other strains. The production of hemolysin is well correlated with virulence (its ability to establish an infection), it is cytotoxic for leucocytes, and inhibits phagocytosis and chemataxis. The *B* hemolysin is cell bound and released only after cell lysis. The production of hemolysin is associated with certain "O" groups and have serum resistance and interfere with host defenses making these much more effective weapons.

Disease is caused by the invasion of the intestinal epithelium in some strains while others simply produce enterotoxin that is absorbed from the site of villus colonization. In either case, the toxins cause the observed symptoms and injury.

When E. coli with K1 antigens successfully invade the blood stream and cause septicemia or meningitis, the mortality rate is very high (40-80%) depending on the circumstances. [The K1 antigen is immunologically identical to group B meningococci]. Those that survive usually suffer permanent neurological or developmental abnormalities. This commends its use in piercing or other physical penetrating and aerosol types weapons in combination with other toxins and organisms. The addition of Iron dextran to virulent strains of E. coli will increase its invasive abilities.

A separate means of identifying or grouping potential E. coli weapons is by the type of epidemiology as follows -

Type	"O" serogroup
Enterotoxigenic	8, 25, 78, 115, 128
Enteropathic	18, 20, 25, 26, 28, 44, 55, 86, 111, 112, 114, 119, 125, 126, 127, 128, 142
Enteroinvasive	28ac, 112ac, 124, 136, 143, 144, 152, 164

The enteroinvasive E. coli share many traits and antigens with Shigella and produce a similar dysentery however it requires about 1-10 million cells to cause the same level of infection and symptoms that only about 200 cells of Shigella dysentery causes.

E. coli also produce **neurotoxins** that can be recovered from almost all strains and used by themselves in biological ordnance. In addition, almost all species and strains of gram negative bacteria produce **endotoxins** (lipopolysaccharides) that can be harvested for weapons. The endotoxins will be covered separately for all gram negative organisms in the next section.

In 1929, Vincent reported that all E. coli strains produced neurotoxins when grown in simple broth at pH 7.6 (much higher than human intestinal pH) at 38 C. A thermolabile exotoxin appears in the medium after 23 hours to five days incubation which lyses at 75 C in 1 hour.

A second toxin appears after about 15-20 days in old cultures in which bacterial lysis takes place and the pH exceeds 10. This enterotoxin is thermostable and is separated out from the exotoxin by heat treatment.

The first toxin produced (the exotoxin) was injected into lab animals producing paralysis which spread from the hindquarters to the front legs, bulbar paralysis, and muscle flaccidity. Twelve exotoxins produced from different strains of E. coli all produced the same symptoms which included apathy, anorexia, hypothermia, early and severe diarrhea, pronounced weakness, and in some animals resulted in coma. Most animals died in 12-46 days. Late and lethal paralysis was the final cause of death.

Vincents research also showed that small amounts of these toxins produced in the colon of humans accounted for various neurological disorders such as narcolepsy, polypnea, torpor, paralysis, coma, cataleptic states, and extreme hyperkinesia followed by death. These symptoms could be treated with serotherapy and this was applied in psychiatric treatments. Given that these toxins can be artificially produced an purified from broth allows for their use as neurological weapons designed to cause mental disorders in the target populations or individuals.

In 1938, Baruk found that not all strains produced the neurotoxin while some quickly lost their ability to produce it and these strains toxin lost potency quickly in storage. He also observed that the toxin may be very slow to act taking weeks or months to produce transient cataleptic and catatonic states. Intravenous inoculation usually produced death before these states occurred, while oral administration caused the longer term "colibacillary psychoses". It was soon found that the correct serological type of toxin needed to be identified in order to treat the toxic infection with the correct antitoxin. When the toxin is used by itself as a weapon, combined with the delay in symptoms, treatment may be a practical impossibility.

The toxins are purified by allowing 3 days of maceration at room temperature. The chloroform autolyzates of bacteria cultured for 18 hours were centrifuged and the supernate, containing a mixture of thermostable endotoxin and thermolabile toxin were treated with cold trichloroacetic acid bringing the pH to 3.5. The acid insoluble thermolabile toxin precipitated while the exotoxin remained in solution. The acid insoluble toxin is immediately redissolved in alkaline solution at a pH of 8.5 and preserved under sterile conditions. It was further purified by gel precipitation, electrophoresis and ultracentrifugation. The crude preparation is concentrated enough for most types of bio ordnance. If the solution does not precipitate but yields a milky suspension, adding 5% sodium chloride and chilling to 4 C should yield a gelatinous deposit which is a mixture of endotoxin, neurotoxin and lipopolysaccharide (LPS).

The crude thermolabile neurotoxin has a nitrogen content of 10.5-13.5%, and a lipid content of 5-10%. Those E. coli strains isolated from urine produced more toxic material than that yielded by intestinal strains. The urine strains produced MLD of .007 mg N (nitrogen) when injected intraperitoneally in mice while intestinal strains produced MLD's of app. 1.75 mg N. Intravenous injection resulted in MLD almost five times more lethal.

Patients without urinary tract E. coli infections did not produce neurotoxin in samples tested. Toxin produced from infected patients always yielded toxin that produced cytopathogenic effects that were irreversible in tissue cultures. It was found also that the urinary tract infection did not induce psychiatric states but that individuals with massive coliform bacilli intestinal infections were found in hospitals exhibiting bizarre behavior patterns and most of these were successfully treated with antibiotics which eliminated the infections and resulting circulating toxin.

Protective Measures

Drinking water is purified by adding .5-1 part per million of chlorine which is an effective bactericide in most cases. Many antibiotics are effective including sulfonamides, ampicillin, cephalosporin, carbenicillin and tetracyclines. Nitrofurans and nalidixic acid have been favored for urinary tract infections.

Most E. coli are killed by exposure to 60 C in 30 minutes and this temperature is used to sterilize materials. Pasteurization of foods and liquids is recommended for suspect substances and for infants who have little immunity.

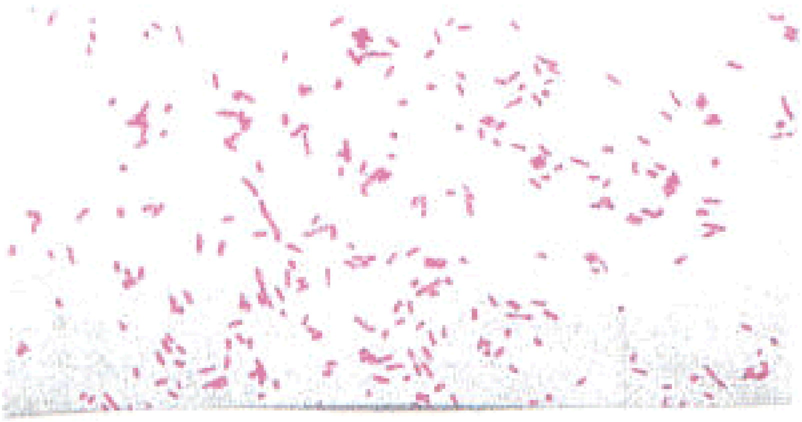
In hospitals, especially with newborns it is recommended that mothers breast fed to avoid E. coli contamination on the milk bottles and also passing on mothers antibodies to provide some antigenic protection.

E. coli colonize the intestine and other tissues by using "adhesins" that allow them to attach to host cells. Antibodies to these adhesins are what normally provide protection against infection and is the bodies principal means of defense. The use of immunization with enterotoxin appears to provide protection against both the toxin and the enterotoxigenic E. coli as well.

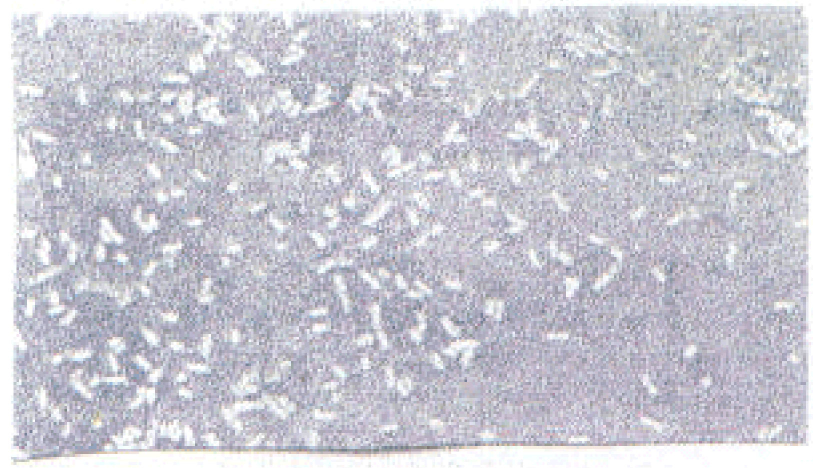
E. coli is susceptible to most antibiotics but their are drug resistant strains becoming prevalent in the US.

Incorporation into Weapons

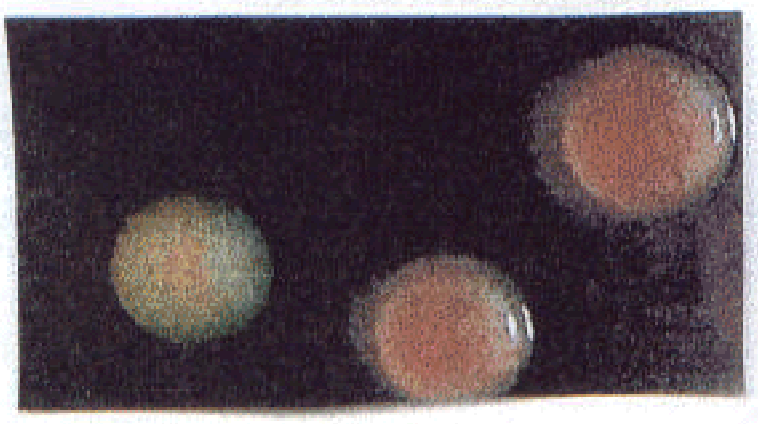
Gram negative E. coli smear of average length and diameter.



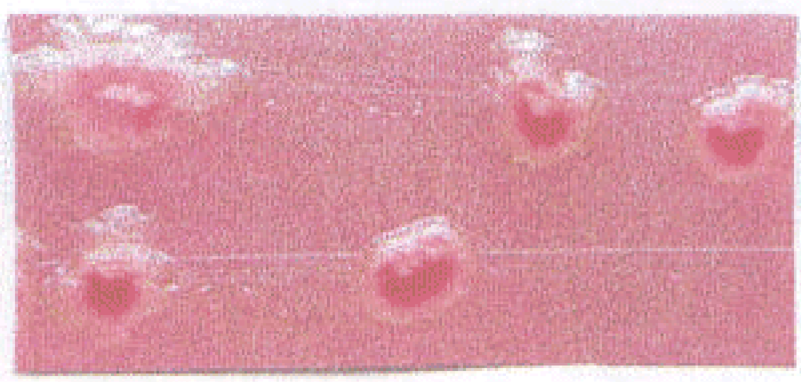
All bacteria cells are colorless. Using Nigrosin stain, the background is filled making the E. coli rods easily seen.



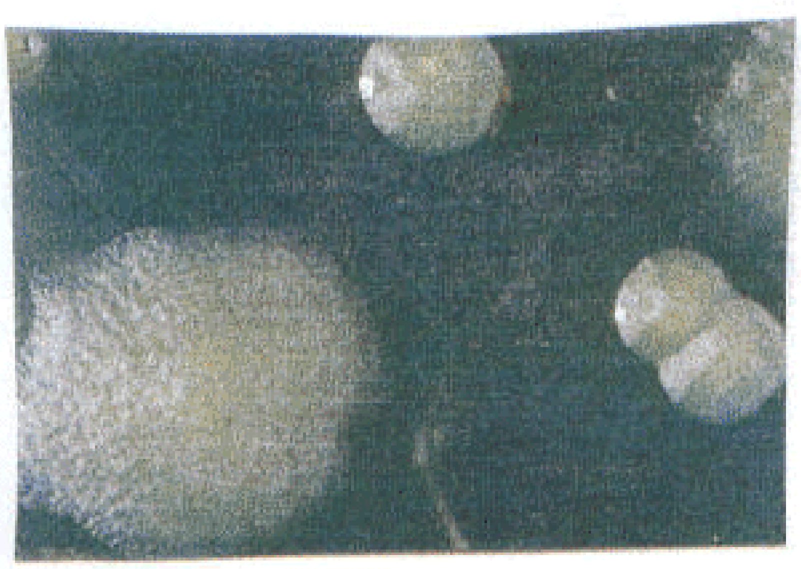
Lactose fermenting E. coli on MacConkey agar in pink while Salmonella is creamy white.



E. coli on MacConkey that are rougher and more opaque due to precipitation of bile salts under the colony (which is why the colony edge is clear, it hasn't had time to act on the bile-maple leaf appearance is easily seen here).



E. coli smooth colonies repeatedly subcultured become rough due to loss of sugars in the O antigen. Start and end subcultures shown together on this plate (same strain).



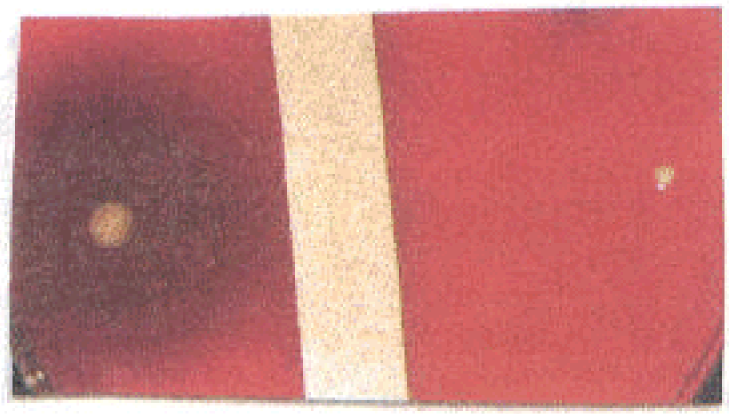
Unusual E. coli that at first does not ferment lactose but then mutates in colony growth to a form that does with the resulting pink spots from bile precipitation around the mutant cells.



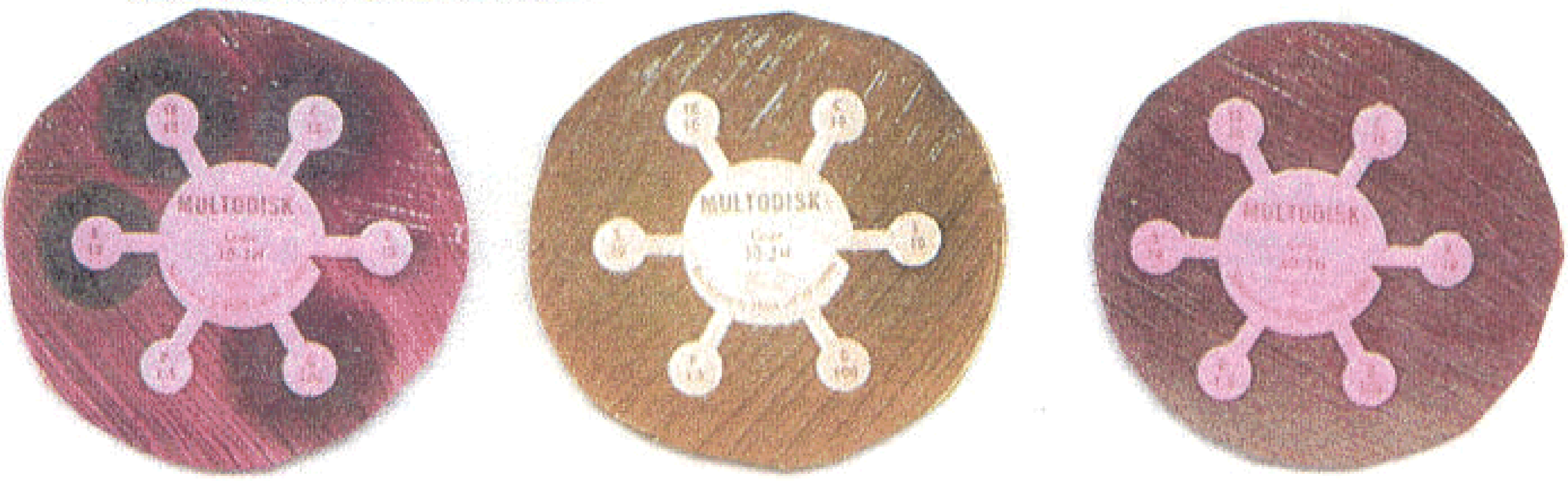
Sometimes a bactriophage (virus) infects a culture and kills the colony cells. Colonies where the "phage" becomes virulent show a "bitten" appearance as seen below.



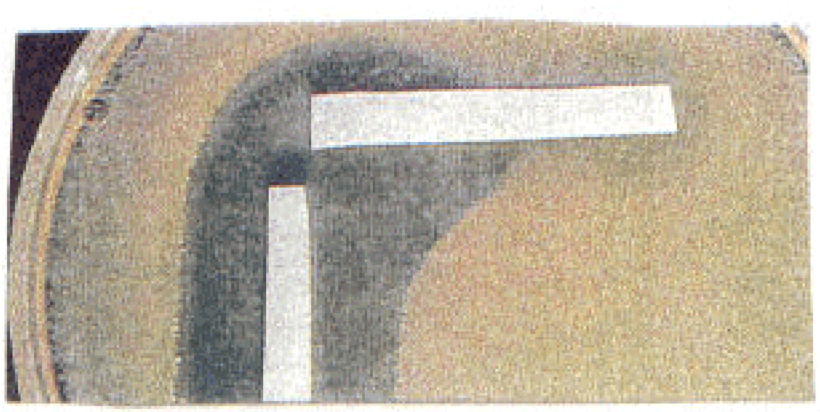
Potassium nitrate was added to the paper strip and placed on this culture plate. The E. coli on the left "browned" the blood agar around the colony showing it reduced the nitrate to nitrite.



E. coli (left) that is killed by several antibiotics. Salmonella typhi (center) that is resistant to all the anibiotics. When grown together in broth, conjugation occurs with some of the E. coli cells receiving drug resistance from the S. typhi. This is a quick way to create and subculture drug resistant strains as seen on right with newly resistant E. coli found and selected from the mixed broth.



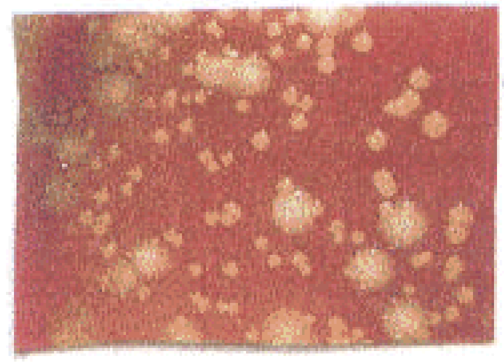
Multiple antibiotics act with greater effect when used together as seen here with E. coli inhibited the greatest in the area nearest both strips which are impregnated with trimethoprim (top) and sulphafurazole (bottom). By subculturing repeatedly from the cells closest to the clear zones, drug resistant mutants can be developed, especially when genetically altered with ultraviolet light or nitrogen mustard.



Blood agar showing gray, opaque, somewhat mucoid colonies typical of the enterobacteriaceae,



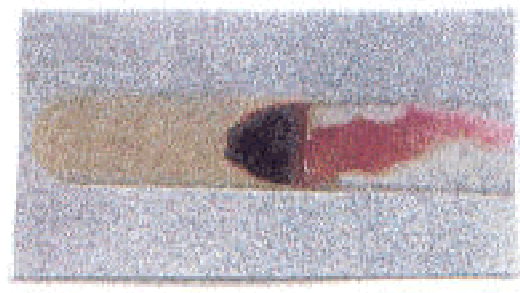
Blood agar with large, mucoid, gray colonies of Klebsiella and smaller, convex smooth, pale yellow colonies of S. aureus.



Blood agar with swarming Proteus seen as sequential waves. Surface is often covered with a thin, nearly invisible layer.



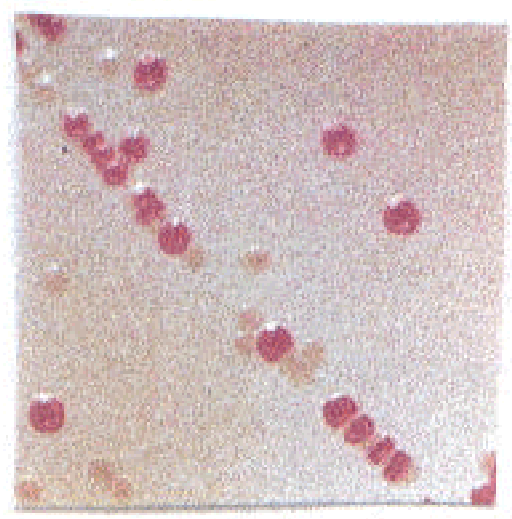
Nitrate test showing positive reaction after reagents are added (reduced to nitrites = red) All enterobacteriaceae reduce nitrates.



MacConkey agar after 24 hrs with E. coli. The red indicates acid production from lactose fermenters.

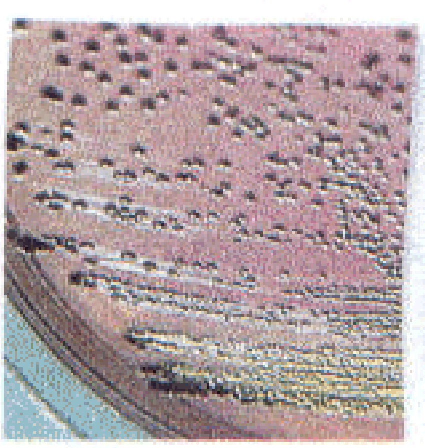


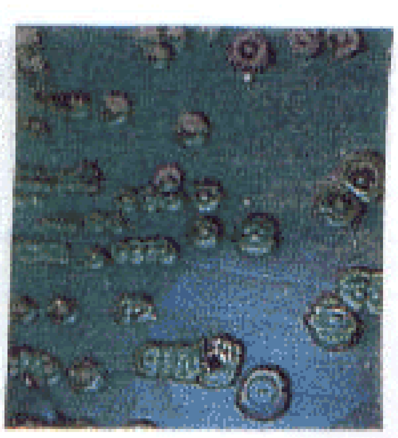
MacConkey with lactose fermenters- red and opaque (non-lactose fermenting) colonies



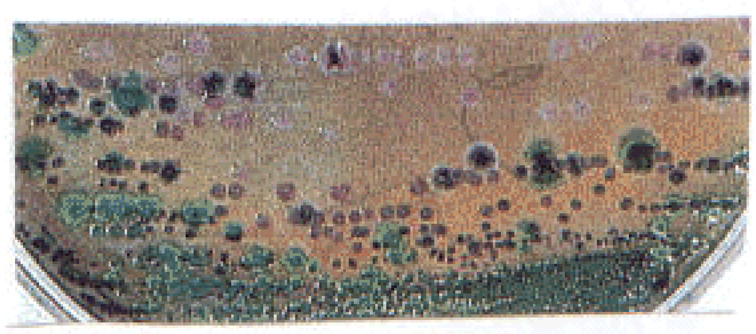


EMB plates with green metallic sheen produced by E. coli which gives strong acid production and a pH drop below 4.5.





EMB with mixed cultures of green (lactose fermenters) and light purple (non-lactose fermenters)



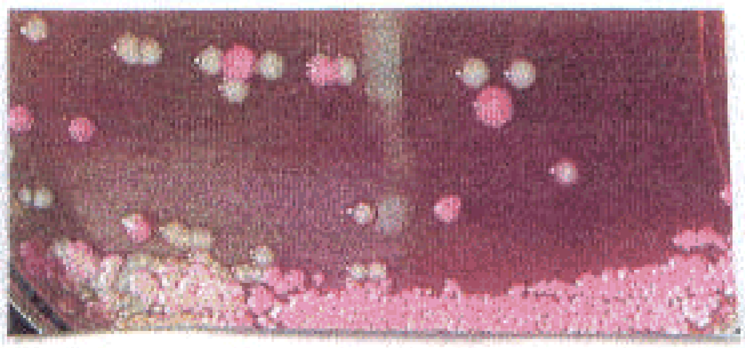
DCA with pink tinge of weak lactose fermenting colonies of enterobacteriaceae.



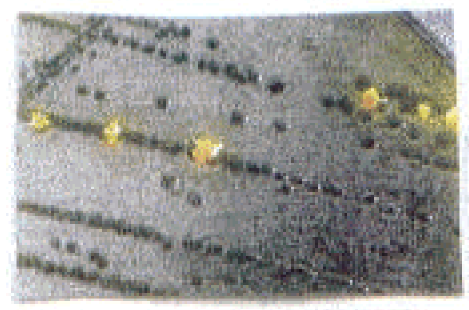
Mixed colonies of lactose fermenters (pink) and many non-lactose fermenters.



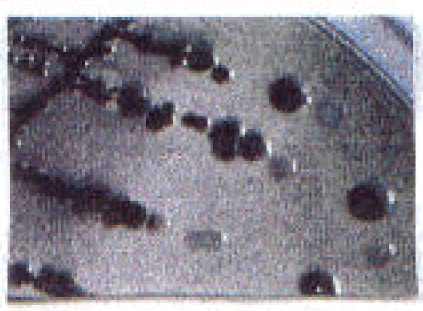
SS agar with red lactose fermenters and gray-white non-lactose fermenters.



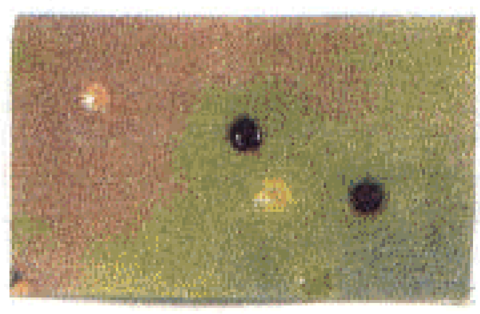
HE plate contains three carbohydrates. Fermenters of any of these appear yellow while non-fermenters appear green.



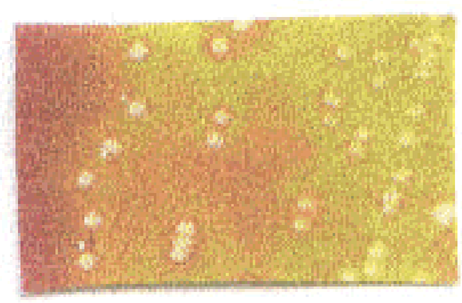
HE plate with green, yellow, and black colonies. Bacteria producing H2S yield black colonies.



Black and semi-transclucent colonies on HE agar.



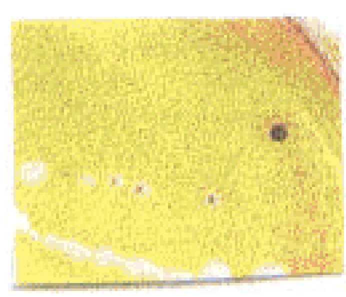
XLD with lactose fermenters change media from red to yellow.



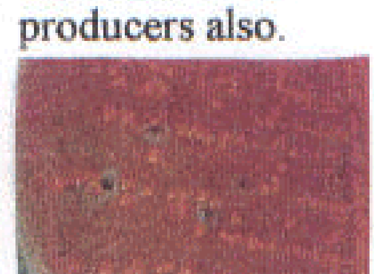
XLD agar with non-fermenters that appear transclucent with black centers (Salmonella-Proteus)



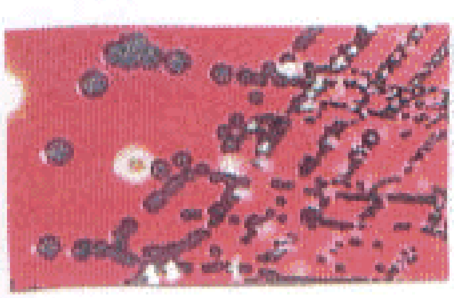
Single deep black colony with strong H2S production (Proteus)



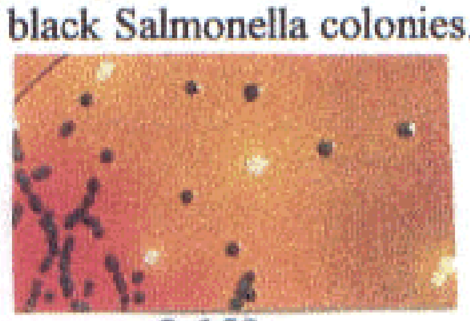
XLD with orange-yellow weak fermenters. Some H2S producers also.



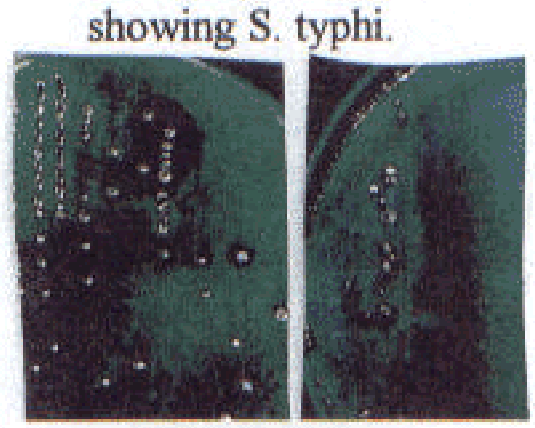
XLD with strong H2S producers and some lactose fermenters.



Pale yellow E. coli and black Salmonella colonies.



Bismuth sulfite showing S. typhi.



Fermentative Gram Negative Bacteria

Gram negative bacteria that ferment sugars (carbohydrates) fall into a large group of organisms called the "Enterobacteriaceae". As a group, these bacteria have caused a great deal of morbidity and mortality as well as affecting almost every human on the planet during opportunistic infections.

These bacteria all grow on similar media and are differentiated by the different enzymes they produce. These enzymes direct the metabolism along one of several pathways that can be detected in growth media and by various testing methods. The enzymes they produce can be detected by various indicators that sense the decay in the media or the presence or absence of the specific metabolic products. From these reactions a biochemical fingerprint for each organism can be made.

Almost all members of this group show the following characteristics-

1. They ferment sugars. When the various sugar is fermented, they produce acid end products. The different sugars are added to the growth medium (milk sugars from dried milk work for general growth). All these organisms ferment glucose by the same pathway producing pyruvic acid and other end by products. This acid production can be detected in the medium by adding indicators.

2. They produce acid from the sugars and this can be visually observed by adding various indicators to the medium so that the pH change is seen as a color change. This is accomplished by adding fuchsin, methylene blue, phenol red, or bromcresol purple to the growth medium and observing the color change around the colonies as they produce acid. Heavy acid producers create large zones of color changes around the colonies.

3. Almost all enterobacteriaceae will take nitrates it finds in the medium and reduce them to nitrites when grown anaerobically. In order to measure this, any nitrate salt like potassium nitrate (at .1%) can be added to the medium. As the culture grows, it produces the enzyme nitratase which breaks down the nitrate into nitrites and water. Any home water testing kit used for detecting nitrites can then be used to tell if the culture medium contains nitrites. Usually two reagents are added (such as napthylamine acetic acid and sulfanilic acid - in that order) to a colony and a red color develops in 30 seconds which means a positive reaction. It is possible that a false negative can occur if the bacteria reduces the nitrites into further breakdown products. For all negative reactions, zinc dust is then added. The zinc will react with any residual nitrates in the medium to produce nitrites and a positive color change when this is done indicates a true negative. This is due to the fact that no nitrates would be left if the bacteria was capable of converting them.

After growing the bacteria and confirming that it produces these reactions, they are then identified by other biochemical methods which will be covered on a species by species basis.

The members of the **Enterobacteriaceae** group are -

Genus	1. Escherichia	E. coli
	2. Edwardsiella	E. tarda
	3. Citrobacter	C. freundi, C. intermedius
	4. Salmonella	S. typhi, S choleraesuis, S. enteritiditis
	5. Shigella	S. dysenteriae, S. flexerni, S. sonnei
	6. Klebsiella	K. pneumonia
	7. Enterobacter	E. cloacae, E. aerogenes
	8. Hafnia	H. alvei
	9. Serratia	S. marcescens
	10.Proteus	P. vulgaris, P. morganii, P. mirabilis
	11.Yersinia	Y. pestis, Y. enterocolitica
	12. Erwinia	E. qmylovora, E. salicis

Only the underlined species will be considered as potential biological weapons agents in this work. Of special note is Yersinia which has previously been classified in the Pasteurella genus. It was known as P. pestis which is the causative agent of the three types of plague - Bubonic, Pneumonic, and Septicemic (also known as the black death).

A number of additives are made to culture media to selectively grow members of this group. Gelatin, which is a complex protein manufactured from animal collagen may be used in many mediums. It changes from a gel to a fluid at 28 C which is why jello is kept refrigerated. Up to now, many of the organisms could be grown on gelatin at the lower temperatures. Use of sodium bicarbonate or other pH buffers added to the gelatin to react with and neutralize acid production is of some use when the bacteria produce end acids.

Many of the bacteria listed above produce gelatinase enzymes which break down the gelatin making it unsuitable as the main growth medium and for this group, agar should be used. It may be desired to identify if the organism in fact produces gelatinase. In this case, the gelatin is added in very high amounts (12%) with the basic materials and the cultures are grown at 35 C to see if the gelatin liquefies. After the growth has occurred, it is placed back into the refrigerator for 2 hours to see if it will resolidify. If not, liquefaction has occurred.

The media used for the selective culture of the enterobacteriaceae include -

MacConkey agar

Peptone	17 g	Agar	13.5 g
Polypeptone	3 g	Neutral red	.03 g
Lactose	10 g	Crystal violet	.001 g
Bile salts	1.5 g	Water	to 1 liter
Sodium chloride	5 g		

Final pH = 7.1

MacConkey is a media used to select and recover enterobacteriaceae and related gram negative species. The bile salts and crystal violet inhibit the growth of gram positive and some fastidious gram negative bacteria. Because lactose is the only carbohydrate, only lactose fermenting bacteria will produce colonies with various shades of red. This is due to the neutral red indicator dye which turns red when the pH drops to below 6.8. The species which ferment lactose and produce mixed acids to lower the pH are E. coli, Klebsiella, and Enterobacter which all produce red colonies surrounded by a zone of precipitated bile.

The bacteria which do not ferment lactose produce colorless or transparent colonies and these include Proteus, Edwardsiella, Salmonella and Shigella. Weak lactose fermenters like Citrobacter, Providencia, Serratia, and Hafnia may appear colorless at 24 hours and become slightly pink in 24-48 hours.

Eosin Methylene Blue (EMB)

Peptone	10 g	Agar	13.5 g
Lactose	5 g	Eosin y	.4 g
Sucrose	5 g	Methylene blue	.065 g
Dipotassium Phosphate	2 g	Water	1 liter

pH adjusted to 7.2

EMB agar is used to separate and isolate different enterobacteriaceae and related coliforms from mixed specimens. The aniline dyes, methylene blue and eosin inhibit gram positive bacteria and fastidious gram negative species. They also combine to form a precipitate at acid pH which identifies acid producers. Sucrose and lactose fermenters are both detected with EMB.

The strong lactose fermenters, especially E. coli produce colonies that are green-black and have a metallic sheen The weaker acid fermenters produce purple colonies in 24-48 hours and include Klebsiella, Enterobacter, Serratia, and Hafnia.

Nonlactose fermenters include Proteus, Salmonella, and Shigella produce transparent colonies while Yersinia which does not ferment lactose, but does ferment sucrose will produce transparent colonies on EMB and purple to black colonies when the EMB is modified with 1 gram of ferric citrate added to the mix.

Desoxycholate Citrate Agar (DCA)

Meat Infusion	375 g	Sodium desoxycholate	5 g
Peptone	10 g	Agar	17 g
Lactose	10 g	Neutral red	.02 g
Sodium citrate	20 g	Water	1 liter
Ferric citrate	1 g	pH adjusted to 7.3	

DCA is used to isolate enterobacteriaceae from mixed specimens. It contains about three times the amount of bile salts (sodium desoxycholate) that are in MacConkey agar. When specimens are heavily overgrown with gram positive bacteria and most coliforms, they are strongly inhibited. The addition of sodium and ferric citrate salts inhibit E. coli and when they grow they appear as small and deep red colonies. The weaker acid producers like Klebsiella and Enterobacter develop mucoid colorless colonies with light pink centers.

Nonlactose fermenters like Proteus, Salmonella, and Shigella grow best producing large colorless colonies and this media is the choice for recovering these strains.

Endo agar

Potassium phosphate	3.5 g	Sodium sulfite	2.5 g
Peptone	10 g	Basic fuchsin	.5 g
Agar	15 g	Water	1 liter
Lactose	10 g	pH adjusted to 7.4	

Endo agar is used to recover specimens from milk, water, and foodstuffs that are contaminated in nature. The sodium sulfite and basic fuchsin inhibit gram positive bacteria. Acid production is detected from a reaction product (acetaldehyde) rather than pH change. It forms a precipitate with the sodium sulfite.

Lactose fermenters appear pink to red. The strong producers like E. coli may change the color in the medium around the colonies and/or produce a metallic sheen from reacting with the basic fuchsin which easily separates the E. coli from other species.

Salmonella-Shigella agar (SS)

Beef extract	5 g	Sodium thiosulfate	8.5 g
Peptone	5 g	Ferric citrate	1 g
Lactose	10 g	Agar	13.5 g
Sodium citrate	8.5 g	Neutral red	.025 g
Bile salts	8.5 g	Brilliant green	.033 g
		pH adjusted to 7.4	

SS agar is highly selective for Salmonella and Shigella and inhibits most coliforms and gram positive bacteria. Salmonella tolerate high levels of bile salts and sodium citrate which is why they are often recovered from gall bladders as a contaminant in salmonella carriers.

Sodium thiosulfate provides sulfur which bacteria can use to produce H2S gas. This is detected by a black precipitate formed with the ferric citrate. Lactose fermenting colonies are colored red by the neutral red while salmonella will appear colorless with black centers from the H2S. Shigella may be inhibited but those that grow produce colorless colonies with no blackening. Motile strains of Proteus do not swarm on SS agar.

Hektoen Enteric agar (HE)

Peptone	12 g	Sodium thiosulfate	5 g
Yeast extract	3 g	Ferric ammonium citrate	1.5 g
Bile salts	9 g	Acid fuchsin	.1 g
Lactose	12 g	Thymol blue	.04 g
Sucrose	12 g	Agar	14 g
Salicin	2 g	Water	1 liter
Sodium chloride	5 g	pH adjusted to 7.6	

HE agar is used to recover high yields of salmonella and Shigella directly from fecal specimens. Acids can be produced from three carbohydrates and acid fuchsin reacting with the thymol blue produces a yellow color when the pH is lowered. When H2S is produced it forms a precipitate with the ferric ammonium citrate.

Rapid lactose fermenters like E. coli is moderately inhibited and produce bright orange to salmon pink colonies. Salmonella colonies are blue-green with black centers from H2S gas production. Shigella appear more green than salmonella with the color fading to the periphery of the colony. Proteus produces small colonies that are transparent and more glistening or watery.

Xylose lysine desoxycholate agar (XLD)

Xylose	3.5 g	Phenol red	.08 g
Lysine	5 g	Agar	13.5 g
Lactose	7.5 g	Sodium desoxycholate	2.5 g
Sucrose	7.5 g	Sodium thiosulfate	6.8 g
Sodium chloride	5 g	Ferric ammonium citrate	.8 g
Yeast extract	3 g	Water	1 liter
		pH adjusted to 7.4	

XLD agar allows for recovery of Shigella and other coliforms. Lysine positive organisms like most S. enteriditis produce yellow colonies from xylose utilization that change to red from lysine decarboxylation. H2S production yields black centers in the colonies.

E. coli and Klebsiella-Enterobacter that use more than one carbohydrate produce bright yellow colonies as do many species of Proteus. Most Salmonella and Arizona produce red colonies with black centers. Shigella and Providencia as well as many Proteus use none of the carbohydrates and produce translucent colonies, the Proteus also having black centers. Citrobacter colonies are yellow with black centers.

Bismuth Sulfite agar

Beef extract	5 g	Bismuth sulfite	8 g
Peptone	10 g	Brilliant green	.025 g
Glucose	5 g	Agar	20 g
Disodium phosphate	4 g	Water	1 liter
Ferrous sulfate	.3 g	pH adjusted to 7.5	

Bismuth sulfite agar is highly selective for Salmonella typhi (cause of typhoid fever). When the glucose is fermented, the sulfite in the medium is reduced with the production of iron sulfide that yields black colonies. It has no shelf life and must be used on the day it is made.

Lactose fermenting coliforms, Shigella, and gram positive bacteria are all inhibited. The S. typhi appear black with a metallic sheen. Most S. enteriditis are black without the sheen. Other Salmonella species produce greenish colonies. A brownish discoloration of the agar many times the colony diameter may be seen.

Enrichment Broth's

Specimens may have very low numbers of disease causing Salmonella or Shigella species and a group of enrichment broth's has been developed to aid in the recovery and growth of these species from fecal specimens. There are often 10 million or more E. coli per gram of feces while Salmonella or Shigella may number as low as 200 per gram.

Enrichment broth's inhibit the growth of the E. coli and other species while allowing rapid growth of SS species. The broth's must be subcultured to fresh broth every 6-12 hours so that the inhibited organisms do not catch up in growth.

Selenite Broth

Peptone	5 g	Water	1 liter
Lactose	4 g	adjust pH to 7.0	
Sodium selenite	4 g		
Sodium phosphate	10 g		

Selenite broth is used to isolate Salmonella from samples of animal manure, human fecal matter, urine, or sewage. It inhibits E. coli and most other coliforms including most Shigella. The medium works best anaerobically and pour depth should be at least two inches.

The broth becomes cloudy in a few hours after inoculation. Subculture is made in 8-12 hours and then this subculture is subcultured to SS or Bismuth sulfite agar in another 8-12 hours. If the broth is overheated during preparation, it may produce a visible precipitate making it unsatisfactory to use.

Gram negative broth (GN)

Polypeptone peptone	20 g	Sodium desoxycholate	.5 g
Glucose	1 g	Dipotassium phosphate	4 g
D-mannitol	2 g	Monopotassium phosphate	1.5 g
Sodium citrate	5 g	Sodium chloride	5 g
Water	1 liter	pH to 7.0	

GN broth allows most Shigella to grow as well as Salmonella. The broth becomes cloudy in 1-2 hours and subculture to HE or XLD agar in 4-6 hours is recommended.

Gram Negative Bacterial Endotoxins

In 1856, Panum took a mixture of decomposing feces, blood, brain and other tissues and filtered them until he had a clear solution and distilled off the liquid. The active material in the residue that remained could be redissolved in water but not in alcohol. After boiling for 11 hours he found that the poison would kill a dog in as little as 100 mg and produced fever at 1 mg. What he had observed was the first experimental production of bacteria produced "endotoxin".

It is now known that most gram negative bacteria cells that are killed yield a poisonous substance that can be extracted and concentrated. It withstands boiling and when injected into animals (especially mammals) produces striking and varied pathological effects. The material came to be called endotoxin because it was believed to be contained inside the cells and was released only when the cells died and partially decomposed (lysed) releasing its internal contents. It was also found to be different from the typical protein toxins in that it was not neutralized by antiserum and did not convert to toxoid by aging or treatment with formaldehyde.

It was also observed that the endotoxins produced nearly identical symptoms when injected into animals regardless of the original species of bacteria that it came from. E. coli, Salmonella, Shigella, and most other members of the enterobacteriacaea all produced a huge range of endotoxins that were biologically related and similarly produced.

Recovery and Production

Bacterial endotoxin is always recovered from cultures of growing enterobacteriacaea in small amounts that are naturally released into fluid culture medium. Heating the cells to damage or destroy the cell walls was the earliest method of releasing the much larger volumes contained inside the cells themselves. Good yields were also obtained by extracting the toxins in hypertonic sodium chloride and sodium citrate. These would solubulize the toxins and allow them to pass through the cell walls without the complete destruction of the walls themselves. By 1950, aqueous phenol had become the most popular method of extracting toxin from the cells. The use of phenol permits the recovery of protein free aggregates of endotoxin that is partially purified.

All gram negative bacteria produce a plastic layer in their cell walls. One of the constituents of the cell walls are "Lipopolysaccharides" (LPS). These are found together in complexes with proteins and phospholipids to form the "O" antigens described in the E. coli in the previous section. All the enterobacteriacaea have O antigens which are complexes containing LPS. These LPS components represent thousands of biochemically different toxins from the thousands of different strains that are recognized as different serotypes. Each organism produces one distinct, specific LPS which accounts for their biological activities as O-specific antigens or endotoxins.

Because the LPS's form in the cell walls, they are part of the intact cell layers and are usually not released into the medium until the cells die and the components begin to decompose. It has been determined that the LPS's are bound to the cell wall by mostly physical forces (ionic, hydrophobic) and can therefore be separated by physical means.

Heat treatment of cell suspensions with sodium chloride solutions or using the chelating agent EDTA (ethylenediamine tetracetic acid) results in the release of about 1/2 of the total LPS complex in a water soluble form. The remaining LPS is bound to the cell wall through metal ligands.

Other methods of extracting LPS involves mild treatment with diethylene glycol, or 50% aqueous pyridine or glycol. By using phenol, formamide, alkaline ethanol, or acetic acid, this isolated complex can be separated into its component parts.

LPS is easily obtained by treating dried bacteria or bacterial cell walls with 90% phenol and 10% water at 68 C for 5 minutes. After cooling, the water phase is separated and it contains LPS, nucleic acids, and other polysaccharides. It may also contain K and Vi antigens. To remove phenol, the solution is either dialyzed or extracted with ether and then repeatedly subjected to ultracentrifugation. This results in a sediment that removes 1-4% of the dry bacteria with the desired LPS and nucleic acids in the supernate fluid. The nucleic acids are usually removed using ribonuclease.

The use of sodium dodecylsulfate or dimethylsulfoxide is also effective in extracting LPS from bacterial cells.

A mutant E. coli that requires lysine has been found to produce large volumes of LPS which it excretes when it is deprived of lysine and has been used in commercial production of LPS.

LPS and Virulence

The LPS forms the O, K and Vi antigens on the outside walls of many of the enterobacteriacaea and this makes them biologically related. E. coli, Salmonella, and Shigella are the most commonly encountered species competing with their individual cell wall antigens in the fecal flora of man and the other primates. The most successful is E. coli which colonizes all primates and humans, as well as most other mammals. Salmonella causes disease and parasitizes many vertebrate hosts. Shigella is the least successful in that it only occasionally colonizes man and the other primates. All are closely related genetically and biochemically.

This genetic similarity allows for transfer of drug resistance via plasmids as described in the E. coli section previously. It is also known that the arrangement of sugars in the LPS of these species has a great impact on the virulence of the individual strains. The most successful arrangement is that of the Salmonella species which can cause a wide range of disease conditions that will be covered in a later section. Those species with fimbrae, filamentous protein appendages that increase bacterial adhesiveness to cells are among the most invasive and virulent. Most of the Salmonella have these while many E.. coli strains have them and only a few Shigella contain these structures. There are many strains that do not have these structures that are extremely virulent. In addition, it is possible to culture strains together to form virulent hybrids from noninvasive strains.

Toxicity

When endotoxin is administered, it produces distress progressing from diarrhea and ataxia to death at a rate which depends on the amount, route of administration, and species of animal tested. Mice are about 1,000 times more resistant than rabbits to equivalent dosages. Resistant and susceptible strains of mice have been genetically bred for toxicity testing of endotoxins and this best illustrates that individuals within a species can vary greatly in susceptibility to endotoxin. Rabbits are considered notoriously variable in this regard.

Man is considered to be the most sensitive species to the pyrogenicity (pyrogen means fever inducing) of endotoxins with rabbits resembling most closely the human response. The ability to affect temperature regulation of the body is directly dose related with small doses (.004 mcg) raising body temperature from 39 to 40 C in about 1-2 hours while much larger doses (.5 mcg) produced fever of up to 41 C and lasting 1-6 hours.

When injected into rabbit skin, it produces local hemhorrhagic necrosis lesions and edema within 2-6 hours. Of special note were early studies where endotoxin was injected into tumors that were normally fatal and induced hemorrhaging only in the tumors. In some instances the tumors regressed resulting in 100% recovery of normally fatal disease. This stimulated a great deal of research in cancer treatment with bacterial endotoxin.

It has been observed that mice poisoned by endotoxin decrease food and water intake in rates proportional to the dose given and even when fed via a tube, the food was not absorbed. Other effects include inhibiting the migration of macrophages, and decreasing the number of circulating platelets by damaging them which induces various damaging host reactions. It was also found that in tissue cultures, it was cytotoxic to normal adult and embryonic tissues while it was not cytotoxic to cells of tumor origin. It has also been observed that endotoxin contributes to arthritis inflammations and induces production of interferon.

It has been found in tests where endotoxins are administered by ingestion that absorption is prevented by bile. When intact cells are ingested in large amounts, the cells that die release endotoxin that is partially absorbed resulting in illness. In 1966, tests carried out (Snell) demonstrated that endotoxin is readily absorbed by aerosols and mimics the effects of injected toxin with the addition of acute interstitial pneumonitis. This commends its use in dust or aerosol based ordnance.

Once animals are exposed to endotoxins (and survive) they become resistant in varying degrees to subsequent exposures. This resistance appears to carry over in varying degrees to serologically unrelated endotoxins. Resistance to the varying effects induced by the toxins also vary. Exposure to different endotoxins induces increasing resistance or tolerance and this has been observed in all animal species including humans.

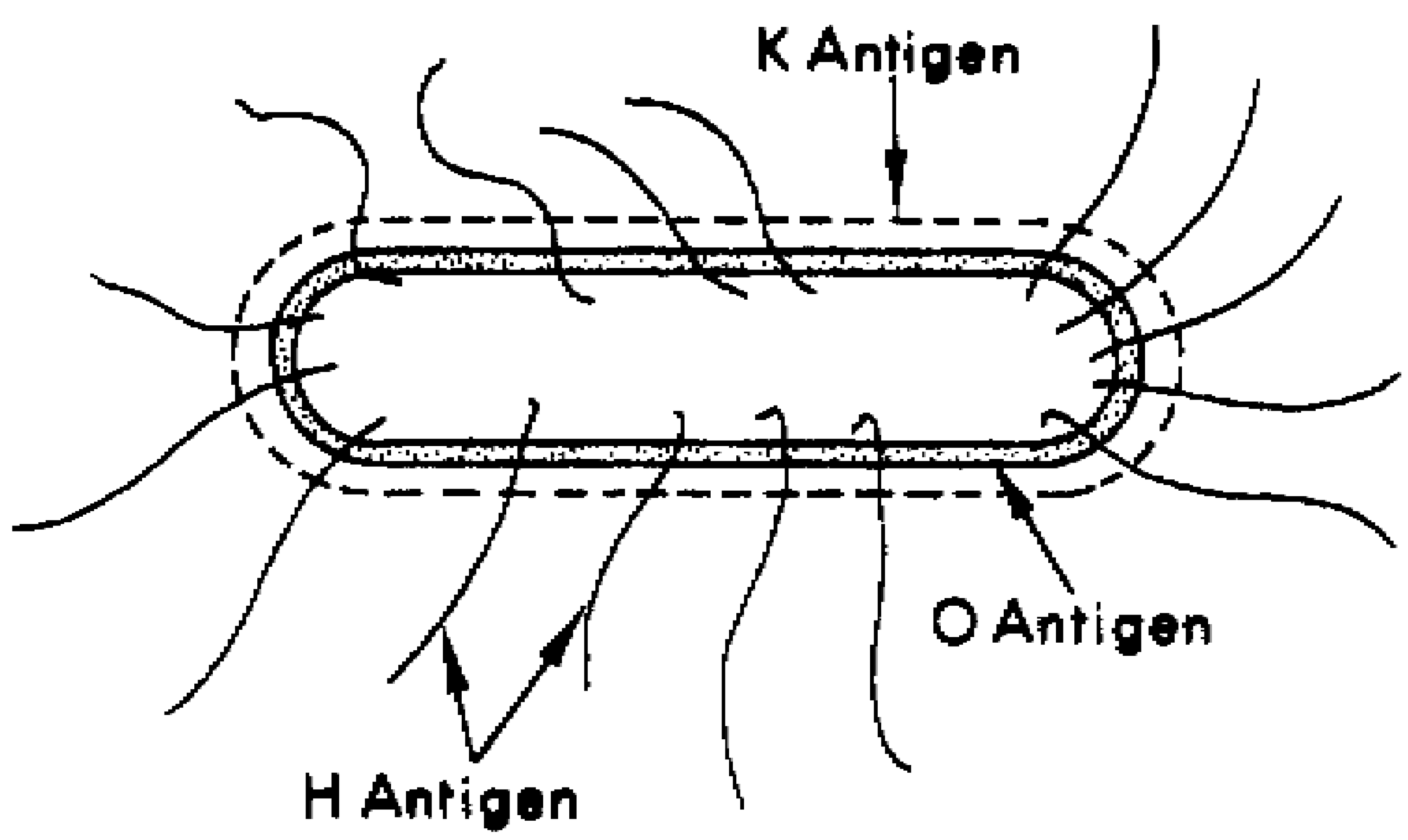
Culture endotoxin grown in blood serum are more pyrogenic than those grown in broth only.

<u>Protection and Resistance</u>

It is possible to treat endotoxins with dilute acetic acid. This splits the endotoxin with a subsequent loss of toxicity but does not cause a loss of serological specificity. 1% solution of acetic acid used to boil the bacilli (or LPS) at 100 C for one hour decreases the lethality by four times. Increasing time of boiling increases the disassociates the LPS at an increasing rate making it more useful in producing resistance to exposure of any of the endotoxins.

It was found that bile or bile salts inactivate the endotoxins but when these salts are diluted out or removed the endotoxins reaggregate to form fully toxic particles.

The frequent injection of progressively larger non lethal doses of endotoxin produces progressively greater tolerance to exposure or attack with endotoxins. The maximum effect lasts only 1-2 months however.



Haemophilus

History and Recovery from Nature

Haemophilus (meaning blood loving) is a group of bacteria that requires special factors from blood before any of its species will grow on lab media. They are strict parasites of man and animals and are most commonly recovered from the throat and upper respiratory tract. App. 30% of healthy children carry H. influenza in their oropharyngeal or nasal secretions and it is also found in the respiratory tract of many healthy adults.

H. influenza was first isolated by Pfeiffer from flu patients during the pandemic of 1889-1890 and was believed to be the cause of influenza until 1933 when the flu virus was finally isolated. This species is now regarded as a secondary invader of the upper respiratory tract. H. parainfluenza is often isolated from the upper respiratory tract of some individuals as a commensal.

H. ducreyi is the cause of chancroid (soft chancres) which is a highly contagious and common form of venereal disease which is sometimes confused with syphilis. The sores or ulcers are exquisitely painful which distinguishes them from syphilis. H. aegyptius is isolated from cases of conjunctivitis (pinkeye) and is the historic Koch-Weeks bacillus. Epidemics are often associated with individuals sharing towels, handkerchiefs or other objects coming into direct contact with the face or eyes. H. aphrophilus is sometimes associated with rare cases of endocarditis, pneumonia, and meningitis. Other Haemophilus species (17+) are isolated on occasion from disease in man but the most significant member of the genus is H. influenza.

The means which H. influenza will suddenly become virulent and cause rapid and life threatening infections in not completely understood although most outbreaks occur in wintertime. Infections in newborns are rare for the first two months after birth and is believed to be due to antibodies acquired maternally. H. influenza meningitis reaches a peak between 2 months and 3 years of age when circulating antibodies are at their lowest ebb. Invasion also occurs with frequency in immunosuppressed hosts receiving chemotherapy. The tissues response produces a suppurative inflammation with an exudate so thick and the edema so severe that it can cause acute airway obstruction requiring an emergency tracheostomy.

H. influenza causes a wide range of respiratory infections which usually precede meningitis after the organism invades the bloodstream.

Haemophilus are aerobic and facultatively anaerobic. They grow best at 37 C but grow from 20-42 C in a pH range of 7.2-7.6. A special set growth factors found in blood and called Hemin and NAD and is required (sometimes both) that are called X and V factors. Hemin (X) is related to hemoglobin and is more readily available to organisms growing on chocolate agar that has been heat treated to release some of the cells components than on regular blood agar. It is heat stable for 30 minutes at 120 C. It can also be derived from a peptic digestion of blood. X factor is required for synthesis of catalase and is necessary for aerobic growth of Haemophilus strains requiring it. Some strains can grow anaerobically without requiring it.

NAD (V factor) is a heat-labile coenzyme which is destroyed at 120 C at 30 minutes. Many bacteria, plant cells, and yeast's produce extra V factor and when these organisms are grown as colonies the V factor diffuses into the surrounding medium. V factor is essential as a hydrogen receptor in the oxidative reduction processes of the organism. With this extra growth factor, Haemophilus species requiring it will grow around the colony as "satellite" colonies. As already illustrated in the Staphylococcus section, Haemophilus can be grown using the staph as a provider of this factor. When Staph produces beta hemolysin, it causes the cells to release X factor into the medium and those species requiring X factor will grow within this zone. By cooking the blood at 80 C for 30 minutes and providing a staph colony, you can provide both X and V factors for all Haemophilus species.

The reason that sheep's blood does not directly supply these growth factors is that during storage, the sheep blood cells lyse and release the enzyme NADase which inactivates any V factor in the media. Rabbit or horse blood does not release this enzyme and can be used to directly grow Haemophilus species but these also grow Streptococci which may make it hard to distinguish between the organisms.

On chocolate agar, Haemophilus colonies reach a diameter of 1-2 mm in 24 hours. They are transparent, colorless and shiny and look like dewdrops. A culture medium can be made called Levinthals medium by mixing tryptic soy broth (500 ml) with 50 ml of sheep's blood and boiling it at 80 C for 20 minutes while stirring. This releases X factor and inactivates NADase. The temperature and time should not be exceeded because the V factor is heat-labile. A deep brown clot forms which is filtered off until you have a clear solution that is sterile. Agar (18 g) is mixed into 275 ml of distilled water and is dissolved by boiling (gelatin can also be used) and is added to 275 ml of tryptic soy broth. Equal parts of both mixes are poured into containers and supplies both X and V factors for growing all Haemophilus species.

Yeast extract which contains V factor, vitamins, and growth factors can also be used in media formulations. It often improves the growth of Haemophilus and can be added to media for maximum production of the organism. V factor is also provided by various vegetable extracts as well.

Haemophilus is recovered from the blood, sputum and cerebrospinal fluid in major infections. Urethral swabs are taken for chancroid and eye swabs for conjunctivitis. Chocolate blood agar with a staph streak to supply the extra V factor is most often used for isolation. The specimen is inoculated first with a single streak run across the plate with the staph. The haemophilus grows in abundance closest to the streak which allows for easy isolation and identification.

Another means of selectively isolating Haemophilus is to add a small amount of Bacitracin, Nafcillin, (or Neosporin) to the heaviest area of inoculation on a plate. This antibiotic will inhibit most other gram positive organisms and allow growth of Haemophilus in many cases.

Differentiation characteristics of Haemophilus species.

	Requires X,V	Affects tissues
H. influenza	X and V	Respiratory tract, conjunctiva and meninges
H. aegyptius	X and V	Conjunctiva
H. ducreyi	X	Genitalia
H. aphrophilus	X	Blood and heart valves
H. parainfluenza	V	Respiratory tract

H. aegyptus can be differentiated from H. influenza because it is soluble in a 1% solution of sodium desoxycholate while H. influenza is insoluble in bile. All Haemophilus species except H. ducreyi reduce nitrates to nitrites which also aids in differentiating from other bacteria species.

In clear gelatin or agar plates which use a peptic digest of sheep's blood as the growth material with brain heart infusion or trypticase soy agar, H. influenza produces small, colorless, mucoid, opaque colonies. Under oblique light, they have an iridescent appearance which indicates they are encapsulated. The nonencapsulated or avirulent strains appear as small, bluish, transparent and noniridescent colonies in oblique light.

Growth and Production

The gram negative coccobacilli are very small, app. .2-.3 *u*m x 1-1.5 *u*m in length. When using gram stain, carbol fuchsin (for 5 minutes) is normally used as the counterstain. In smooth cultures and exudates, small regular forms dominate while in rough cultures and in healing exudates pleomorphic forms are often seen. These range from coccoid (oval) to long filamentous forms. In both broth cultures and cerebrospinal fluid, the long threadlike filamentous forms are the most common. Some of the filaments can reach 30 *u*m or more in length. The ends of the cells are usually rounded.

Haemophilus are nonmotile, nonsporing and usually produces capsules. Pili are produced by most H. influenza and these are responsible for hemagglutination of human type O erythrocytes as well as aiding in adherence to pharyngeal tissues and aid in colonization.

H. ducreyi appear as minute gram negative bacilli which are often clustered intracellularly in the cytoplasm of polymorphonuclear leukocytes while the extracellular bacteria form chains of cells in a "boxcar" arrangement. These organisms can be difficult to culture and often require 5%-10% added CO2 while growing at 35 C for 1-2 days. Some fastidious strains require using human serum or freshly clotted rabbits blood in the specimens and incubating for 24 hours at 35 C before inoculating lab media.

Toxicity and Harm

Haemophilus species have been classified into serotypes based on capsular polysaccharides with 6 types of Haemophilus influenza designated to date. The specific soluble substance is the polysugar phosphate which are designated a through f. The serotype b is the predominant cause of meningitis and respiratory infections and these strains appear to be resistant to the bactericidal effect of complement and are not cleared from tissues in unimmunized hosts. Other antigens are shared by many of the species. In culture media, the smooth organism readily transforms by mutation to the (often avirulent but still opportunistic) rough form which then lacks the specific polysugar phosphate. These strains then become serologically diverse. These rough strains are found in aver 50% of normal throats. These strains are often opportunists causing disease when the respiratory tract is less resistant to invasion by bacteria.

H. influenza can cause primary and secondary infections of varying severity and is often associated with meningitis, chronic sinusitis, mastoiditis, and middle ear infections. Meningitis in infant is associated with very high mortality rates. Those that survive the meningitis often have neurological damage such as deafness, speech impairment and behavioral anomalies. It is known that viral infections favor the development of Haemophilus infections and especially meningitis. Subacute bacterial endocarditis also occurs as an occasional complication.

The cell substance of H. influenza is toxic as it is in many other bacteria. Toxic substances are produced in broth cultures, are filterable and appear in quantity after 6-8 hours incubation. These substances cause ciliostasis and epithelial cell damage. It is believed that the toxins may be endotoxin and that exotoxin is not formed.

H. influenza is not normally pathogenic to lab animals but mice may be infected when injected intraperitoneally with strains suspended in mucin. Only the capsulated strains resist the bactericidal action of normal rabbits blood.

Protective Measures

Haemophilus species are killed at 56 C for 30 minutes and by drying. Most disinfectants also kill them quickly and the tetracycline's chloramphenicol and ampicillins are the most frequently used drugs in treatment. These are used prophylactically at rest homes during influenza outbreaks on those individuals most susceptible to infection.

Haemophilus are also susceptible to chilling and are killed by extended refrigeration.

Immunization with type b capsular polysaccharide results in bactericidal and opsonic activity from antibody response. This reduces the incidence of invasive disease, however children under 18 months of age do not respond to the vaccine and do not acquire immunity or protection.

<u>Incorporation into Weapons</u>

All Haemophilus species are capable of causing infection and disease when they have entered breaks in the skin, are inhaled or become involved in the eyes. In order to grow in these tissues it is important that X and V factors from the boiled (and absorbed on a suitable dust carrier for storage) blood and tryptic soy be incorporated for use in the final weapon. This insures that the surrounding tissues will support the abundant growth of these bacteria.

Almost all cultures die off quickly so the organism must be continually subcultured on chocolate agar or in broth. They may also be preserved by lyophilization or storage in an ultrafreezer.

The best use of this organism in weapons design is in combination with multiple organism in "cocktail" attacks. Animal models have been used for respiratory inoculation with resultant high incidence of meningitis and empyema. The capsular strains resist phagocytosis and are the preferred strains.

Listeria

History and Recovery From Nature

L. monocytogenes is widely distributed in nature. It was first isolated in separate animal outbreaks in 1926. It is part of the normal microbial flora of healthy ferrets, chinchillas, ruminants, foxes, gerbils and humans. It is frequently isolated from abortions in cattle and sheep. In rodents and poultry it is often associated with necrotic hepatitis and myocarditis. It is also found in sewage, fertilizers, decaying vegetation and soils and lives in the plant-soil environment. It has been reportedly isolated from over 50 species of animals (Welshimer) and primarily causes an encephalitis when infectious in nature. It is the most frequently isolated gram positive disease causing bacilli in many labs.

Listeriosis occurs in humans as conjunctivitis, cervicoglandular often with pharyngitis, pneumonic with symptoms similar to typhoid fever, and cutaneous. It also causes genital infection with habitual abortion and perinatal infant septicemia. Epidemics with newborn infants occur with some frequency. Septicemia and meningitis are the most common forms of the illness in older humans who have decreased immunity and host resistance. Use of corticosteroids by the host increases the mortality rate significantly. In cases where Listeria is disseminated throughout the body vie the bloodstream it may involve the formation of multiple, focal, acute inflammatory lesions or abscesses throughout the viscera.

It is a cause of meningoencephalitis in which exudate in the cerebrospinal fluid contains mononuclear or polymorphonuclear exudate with monocytosis. The disease is diagnosed by isolation of the organisms from the spinal fluid. It is often associated with corticosteroid or radiation therapy which indicates that a latent infection already exists and are activated by these treatments. In untreated cases, the mortality reaches about 70%.

Humans can acquire the disease from infected dogs, ingestion of contaminated milk, or infected meat. In the US, livestock and poultry are considered the prime reservoir of the organism. Mortality of listeriosis (blood based) is 42-50%.

Recovery of Listeria in culture can be difficult due to contamination. A cold enrichment technique is recommended in the literature where the collected specimens are refrigerated at 4 C for several days to several weeks prior to inoculation into lab media. [Cultures from sheep and cattle brains <u>must</u> be refrigerated before inoculation for isolation.] This is because the Listeria can grow slowly at this temperature, thereby enriching the inoculum over other contaminants.

On sheep's blood at 35 C for 24 hours, the growth is usually light but if incubated with 5-10% CO2, or anaerobically their is improved growth. Colonies are small, translucent, and gray with most strains producing a narrow zone of beta hemolysis around the colonies which is similar to that of beta hemolytic Streptococci. L. monocytogenes is never alpha-hemolytic and does not form a white pigment which helps differentiate the colonies from other gram positive bacilli. Occasionally, elongated filaments are seen in cultures grown in solid media at room temperature.

L. monocytogenes is catalase positive, is most motile at 25 C, will grow at 4 C slowly, has a narrow zone of beta hemolysis on blood agar, and ferments glucose, trehalose, and salacin. Acid is produced without gas with glucose, maltose and other sugars. It hydrolyses esculin and is H_2S negative. The catalase test is accomplished by adding a few drops of 3% hydrogen peroxide to colonies isolated on heart infusion agar. It produces catalase while Streptococci do not.

Growth and Production

Listeria are short (2 x .5 um), gram positive coccobacillary rods appearing in pairs at an acute angle end to end to one another. Diplobacilli may occur in short chains and in smears they can be found both intra and extra cellularly. They are non-encapsulated, do not form spores, and lose their gram positivity almost entirely in cultures older than 48 hours and may even appear gram negative. If the gram stain is overdecolorized, the cells may appear gram negative and can be confused with Haemophilus. In other smears, they may assume the pleomorphic, palisade forms of diptheroids. In older and in rough culture colonies they may form filaments 6-20 um in length.

In broth cultures grown at 37 C they are mildly motile or sluggish but when grown at 25 C for 6 hours, they display a characteristic tumbling or "head over heels" motility. This motility also provides one of the simplest way of identifying the organism and differentiating it from other gram positive rods like Corynebacterium. In semisolid agar this motility can be seen as an umbrella shape growing into the medium below the colony for 2-5 mm. The organism exhibits up to four flagella when incubated at 25 C but usually has only a single flagellum at 37 C. They grow well on ordinary media at 37 C but show enhanced growth when liver extract, blood, serum, or glucose are added.

Colonies appear early on as tiny droplet-like forms and reach 2 mm in diameter in 2-3 days. They become smooth, transparent and then finally opaque while becoming larger with raised edges. A small zone of beta-hemolysis may be seen on blood agar. Gelatin is not liquefied and is useful for growing this organism at the cooler temperatures. In stab cultures, growth occurs evenly along the length of the stab. On MacConkey agar there is very light growth and on media with tellurite added, the colonies are small, black and glistening with a zone of green color surrounding it. On clear tryptose agar the colonies have a blue-green color in oblique light.

Listeria can also grow at high pH (9.6) and at 10% sodium chloride making it easy to isolate using these characteristics as well. Growth is slow in the absence of fermentable carbohydrates and may not occur at all if the inoculum is small.

Listeria does not produce a classic exotoxin. What it does produce is a substance or combination of substances from culture filtrates that have been described as -

1. MPA-monocytosis producing agent which, when injected into young rabbits produced a circulating monocytosis similar to that seen in Listeria infections in rodents and birds. It is directly associated with virulence of the strain and species.

2. A protein substance from glycine lysed Listeria that was not toxic by itself but had infection potentiating properties called MEF (mortality enhancing factor). It was a heat stable cell disruption product which enhanced the mortality rates of Listeria, Brucella, Proteus, Streptococcus, and Staphylococcus when injected into experimental animals.

3. A highly toxic polysaccharide fraction is obtained from live Listeria cells using absolute alcohol, sodium acetate, and acetic acid.

4. Mechanically disrupted cells yielded a protein fraction that produces disturbances in electrocardiograph tracings, increased respiration rates and changes in blood sugars as well as circulating monocytosis when given intravenously to rabbits.

5. A soluble hemolysin that in early reports did not have any classic toxic properties when injected into test animals. It was believed that it may function as an accessory factor in the invasiveness of the organism. Since then it has been shown to be very toxic to mice and has a leucocidin effect, disrupts lysosomes, and affects cardiac tissue.

Peak production of hemolysin was at 18 days in soy broth culture at 20 C. Peak levels at 37 C are reached in 24-48 hours. 100% of the hemolytic activity is precipitated at 60% ammonium sulfate concentration. The lysin is non-dialysable, heat-labile, inactivated by trypsin, is sensitive to oxygen and is antigenic. It appears to be enzymatic in nature. Strains of Listeria that produce high levels of this hemolysin and are most virulent produce an opacity in egg yolk broth. This suggests that the lysin is a lecithinase.

The cardiac disruption ability of the hemolysin in mince indicates that it can cause ventricular standstill and atrial fibrillation at low doses making it a potent "heart attack" weapon. In sublethal doses, cardiac injury was notable, especially to the pacemaker and contractile tissues.

Antiphagocytic factors are also present in the cell wall of Listeria which aids in its ability to ward off the host defenses. An endotoxin like material has also been finally identified (with difficulty) that is similar to the LPS endotoxins. It is located on the surface of the organism and is highly lethal to rabbits.

All of the above indicates that the toxins act to increase the invasiveness of Listeria and other organism, and affects a wide range of tissues. In addition, all the virulence and toxic factors may be restored or enhanced by passage through susceptible animals.

Freshly isolated smooth colonies are always the most virulent and this virulence can be easily lost in artificial culture requiring passage through a mouse or rabbit for maximum return to effectiveness.

Protective Measures

Mortality rates due to Listeria infections is greatly reduced by treatment with ampicillin, penicillin, erythromycin and tetracyclines. It is usually resistant to sulfonamides, bacitracin, and polymixin making "Neosporin" added to a media surface as another way of isolating it as well.

High doses of antibiotics are necessary for septicemia and meningitis.

Incorporation into Weapons

Listeria is a useful weapon that can attack unprotected eyes as well as initiating high mortality rate inhalation disease. It is a good addition to "cocktail" inhalation and dermal based weapons and is best used in cold, winter weather.

The production and concentration of the various toxins and infection enhancing substances make this organism valuable as a weapon. Its by products will improve the infectability of almost any other organism and can cause direct cardiac damage and death by itself. This organism and its production products may be able to significantly enhance virtually every class of bio weapons by all exposure routes.

Brucella

History and Recovery From Nature

The first Brucella organisms were first isolated in 1887 by Sir David Bruce for whom it is named. The organisms were recovered from the spleen in fatal cases and were traced to goats which transmitted them to peasants and visitors to Malta who drank the goats milk. The disease became known as Malta fever, an undulant fever caused by B. melitensis. Undulant fever in man is a chronic, relapsing febrile illness. It is characterized by weight loss, anorexia, night sweats, and development of granulomatous inflammation of various tissues and bone. Three species of Brucella that originate in animals all cause this fever in humans.

The organism is endemic in and around the Mediterranean and its islands. A large number of goats, sheep herding dogs, and other animals provide a solid reservoir. The brucella enter the body via ingestion, through abraded skin surfaces, the eyes, and the respiratory tract. They enter the blood stream via the regional lymphatics and localize in various tissues throughout the body. Pregnant goats are very susceptible to Brucella and shed the organism in its milk and urine. A generalized bacteremia lasts about one month and then becomes localized in the udder and uterus where the organism can persist for months to several years.

B. suis is found in swine and is responsible for epidemic abortions in this species. The aborted fetuses and placenta are the primary source for recovery of the organism. It is virulent to man but is less harmful in its effects than B. melitensis. B. canis is found in about 9% of stray dogs and occasionally will infect humans. In Alaska, reindeer serve as a host to Brucella, rabbits, waterfowl, wood rats, and lab animals have also been found to carry the organism.

The organisms are intracellular parasites and are partially protected from cellular and immunological defenses as well as antibiotics. For this reason it tends to cause chronic infections. They are the cause of epizootic abortions in cattle, hogs and goats and affect virtually every organ and tissue. The organism can be recovered from bone marrow and various affected tissues, usually associated with the reticulo-endothelial system, and in the fist two weeks of infection are recovered from the blood. The substance erythritol (an alcohol) stimulates the growth of virulent Brucella and is found in the placental tissue of animals which accounts for large organism growth and abortions. Human placenta do not contain erythritol and do not experience the abortion aspect of the disease.

All Brucella species are strict aerobes growing best at a pH of 6.8 to 7.2 and at 37 C. All grow well on simple media but some species require added CO_2 for isolation or best growth. Brucella grown on blood agar are round, semispherical, smooth, and opaque with a white or dull creamy colored hue. The colonies reach 2-4 mm in 24 hours with larger growth on enriched media. Adding serum, liver extracts, yeast hydrolysates, or whole rabbits blood in meat infusions will increase isolation and recovery of the organisms.

Brucella produce H2S and urease. They are catalase positive and this positivity is associated with virulence. They reduce nitrates to nitrites and are cytochrome oxidase positive. It requires culture media with peptone and tryptose or trypticase added for growth plus the enrichments described above. Even with the best growth conditions it may take two to three weeks for colonies to be seen. The blood is often added to trypticase soy broth for enrichment followed by transfer to agar in 4-7 days. Carbohydrates are used but gas or acid production is hard to detect.

A special Brucella agar is prepared by mixing pancreatic digest of casein, peptic digest of animal tissue, yeast autolysate, and sodium bisulfate. It strongly inhibits most other bacteria allowing for selective growth of Brucella. Crystal violet and Bacitracin, or thionin or basic fuchsin dyes are added for heavily contaminated specimens.

Brucella is contracted directly from infected animals or indirectly through milk, water, manure and soil. Lab workers are at highest risk because the cultures easily create aerosols and only a few cells are required to initiate infection. There are many documented incidents of careful lab workers acquiring the disease. Brucella can also be recovered from cheese made from infected milk for several weeks as long as the pH remains close to neutral.

Despite the fact that it is easy to spread, Brucella is hard to initially recover in culture. Repeated cultures of blood are usually required and give positive results only 30-50% of the time. Glucose serum broth is used with one under 10% CO2 and the other without. The organisms are believed to be concentrated in the leukocytes and the sediments of lysed cells are cultured after being recovered in a centrifuge. Trypticase soy broth is used in part of the surface of the broth growths. If tiny colonies appear on the surface in a few days these are examined for appearance and testing. Serum dextrose agar is also sometimes used for growth of Brucella.

<u>Growth and Production</u>

Brucella are very small, non-motile, non-sporing gram negative coccobacilli measuring .6 x 2.2 *u*m in length. They appear as round or oval forms singly, in pairs or short chains in some cultures. Small capsules are sometimes observed. In culture they may appear as either entirely coccoid or bacillary while in exudates or tissues they may be found as both. With frequent subculture, B. melitensis are almost completely coccoid. They grow best at 37 C but will grow at 20-40 C.

In culture, pinpoint colonies that are convex with a smooth, glistening surface usually appear within 3-7 days. Colonies are translucent, amorphous, and convex. It produces no hemolysis or pigment on blood agar. The organism grows best on media with animal proteins such as those already described. In gelatin stabs, a delicate line of growth is seen along the track of the inoculating wire with little or no surface growth. There is no liquefaction on gelatin. On potato medium with several days of growth, a chocolate brown colony appearance is seen.

A thermo-labile protein with cytotoxic properties can be extracted from B. melitensis using trichloracetic acid. It also acts as a mild neurotoxin. The cell envelope also contains an LPS in smooth virulent colonies that is also toxic to the host. Rough colonies are generally avirulent although these strains are capable of intracellular growth. A change of smooth to rough forms in culture usually means a loss of virulence due to the loss of O antigen side chains in the cell envelope. This is due to alanine metabolism in which the S form produces it until it reaches toxic concentrations and then the rough alanine resistant form begins to overgrow the original cells. Oxygen tension also contributes to this conversion.

Lab animals can be infected with the guinea pig being the most susceptible to small inocula. The animal usually recovers from the infection but B. melitensis may be fatal in 6-8 weeks. The lymph nodes are swollen and the spleen may be greatly enlarged and engorged with necrosis seen on the spleen and liver from which the organisms may be recovered and cultured.

Protective Measures

Brucella generally are sensitive to antibiotics but the initial doses must be kept small because the organism releases an endotoxin that causes severe reactions on death. The cells are also located intracellularly which often protects them from antibiotics. As a result, prolonged antibiotic therapy is usually required. Sulfonamides, streptomycin, tetracycline's and chloramphenicol have been the drugs of choice for most strains and rifampin has recently been found to be very effective.

Pasteurization at 60 C of all dairy products and chlorinating water can help prevent Brucellosis. They are moderately sensitive to acid and die out in a few days in cheese undergoing lactic acid fermentation. They survive for several days in butter made from infected milk. Most herds in the US have been rendered Brucella free from rigid government controls but a large reservoir of wild animals carry it.

Brucella are sensitive to direct sunlight and areas can be sterilized with ultraviolet light.

Human resistance to Brucellosis is cell mediated in a manner similar to that of tuberculosis. The immune response does not seem to fight off the infection well once it is established but does offer some protection against acquiring it in the future. Killed vaccines that have been tested produce antibodies but do not protect against the disease.

Incorporation into Weapons

Brucella is one of the most easily acquired infectious organisms. Only a few cells are required to initiate disease and a single culture plate converted to aerosol can literally infect millions. It is possible to infect by all exposure routes making it one of the most potent weapons available. Although usually not lethal, it can incapacitate significant fractions of cities, armies and nations when high infective doses are used.

They survive well on dust carriers (and in soil) and have been recovered from dead fecal material after 2-3 months.

The disease was very costly to the British in both world wars with tens of thousands of man hours lost due to its effects on soldiers on the island of Malta. The morbidity was well documented and many physicians were brought together on Malta to form a commission to aid in its control. Its mortality rate in untreated cases was only 2-3% but its ability to incapacitate an entire army for months is well documented.

In about 10% of patients it has also been noted that neurological and psychiatric disorders occur which adds to its effectiveness as a long term weapon of war.

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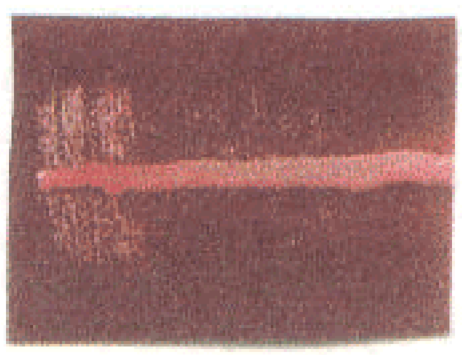
Smear of Haemophilus influenza with some longer forms present with the oval bacilli.



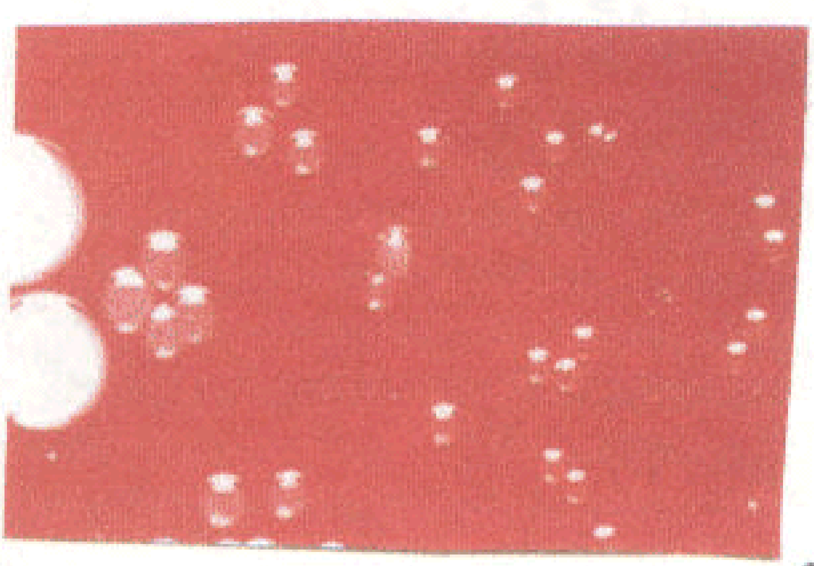
Boxcar arrangement of H. ducreyi.



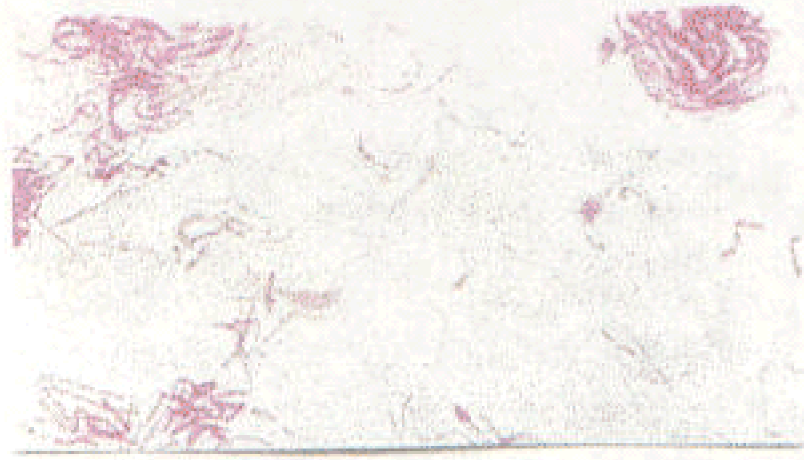
Growth around Staph streak.



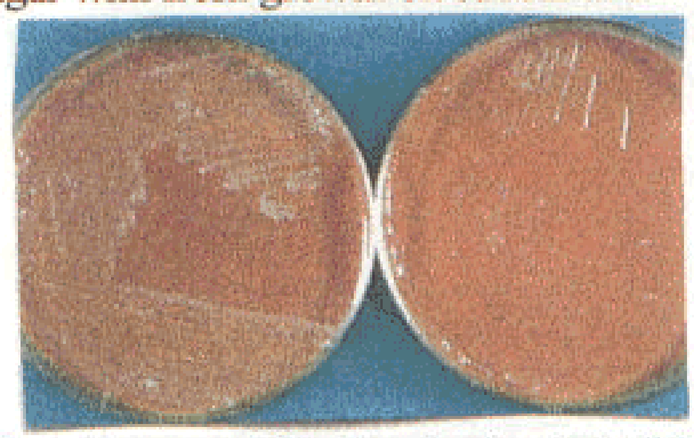
Close up of sattelite colonies diminishing in size with distance from the Staph.



Long filamentous forms of Haemophilus



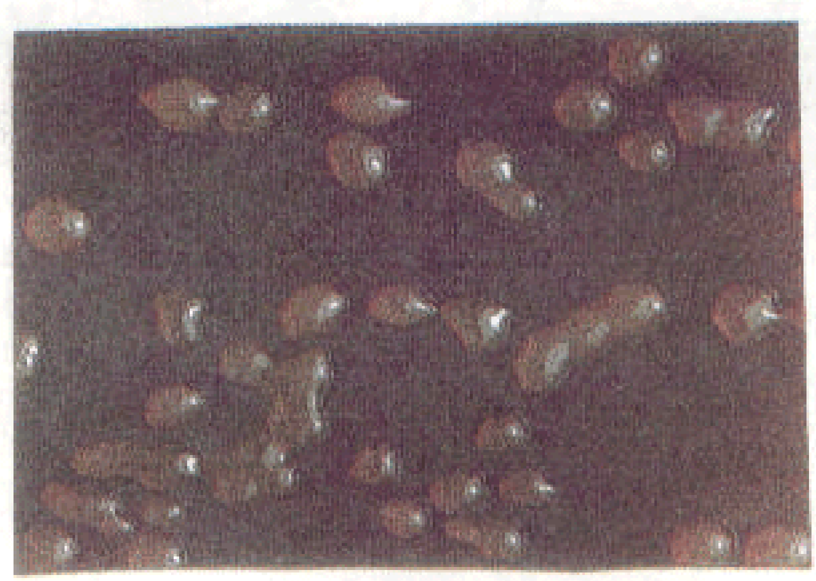
Old, mucoid, growth (48 hour) on chocolate agar with fresh growth on subculture.



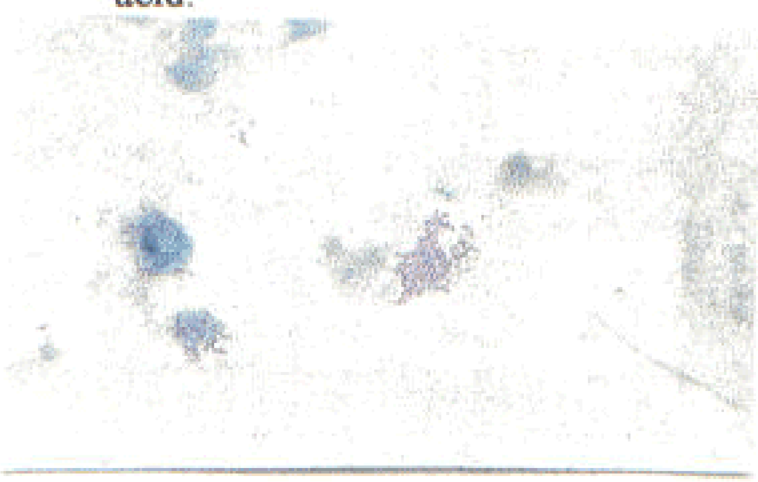
Growth around Staph colonies (sattelitism).



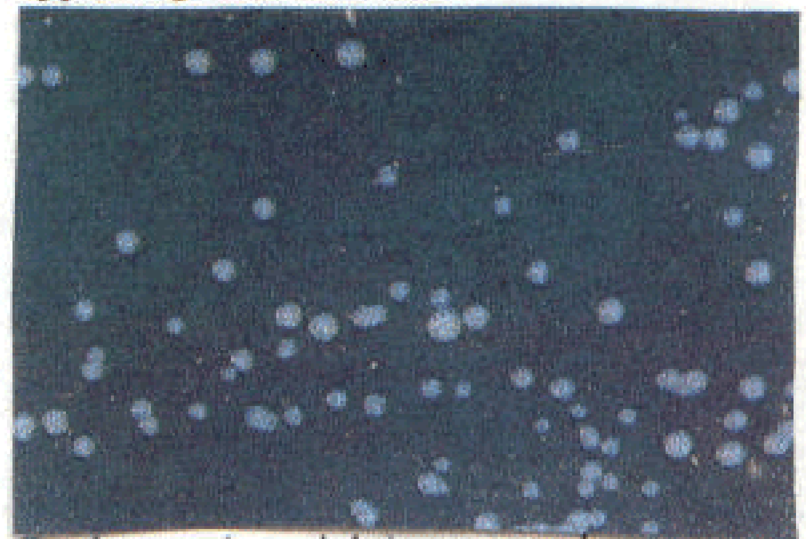
H. influenza type B on medium with treated blood. Large, mucoid (capsulated).



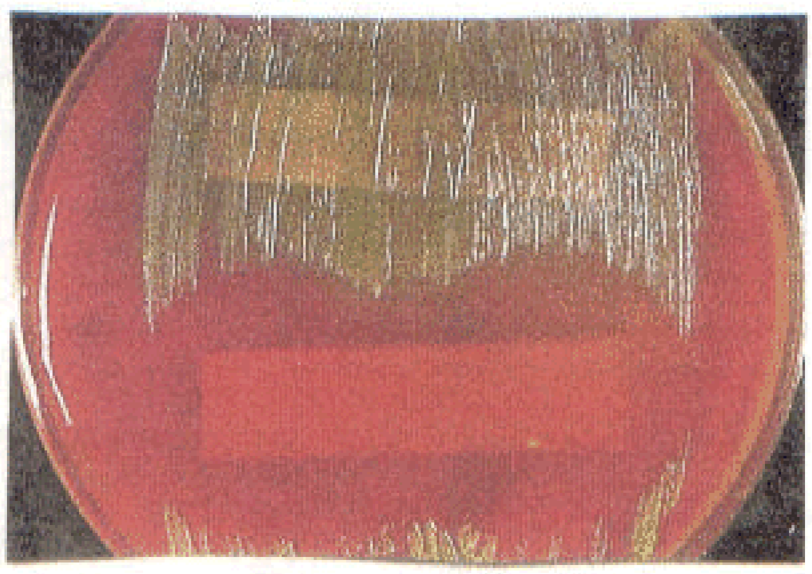
Bovine fetus showing B. abortus. It resists decolorization with weak acid.



Brucella on serum dextrose agar appearing small and blue.



Heavily sown blood agar plate with thionin impregnated strips supporting abundant growth and basic fuchsin inhibiting it.



B. abortus (top right) grows only on fuchsin impregnated serum dextrose agar. B. suis shows no growth while B. melitensis grows on both fuchsin and thionin.



Toxicity and Harm

Humans are very susceptible to Brucellosis. Most infections are subclinical with brucellar agglutinins in the blood of many individuals. There are three phases of Brucellosis, acute, subacute, and chronic.

The acute form has an incubation period of 4-30 days and the classic undulating fever is rare in North America. The symptoms are usually mild (in all phases) but can be disabling because ofd their persistence. Local and focal lesions may appear in the skin, mouth and lung tissues. Rare cases of abortion and pulmonary infection are also known. The acute phase is self limiting within 1-3 months. During the second and third weeks, cultures are most likely recovered that can be positive.

The subacute phase can be more or less severe and last for several months. When it lasts for over a year it is considered chronic and in some cases has persisted for 20 years.

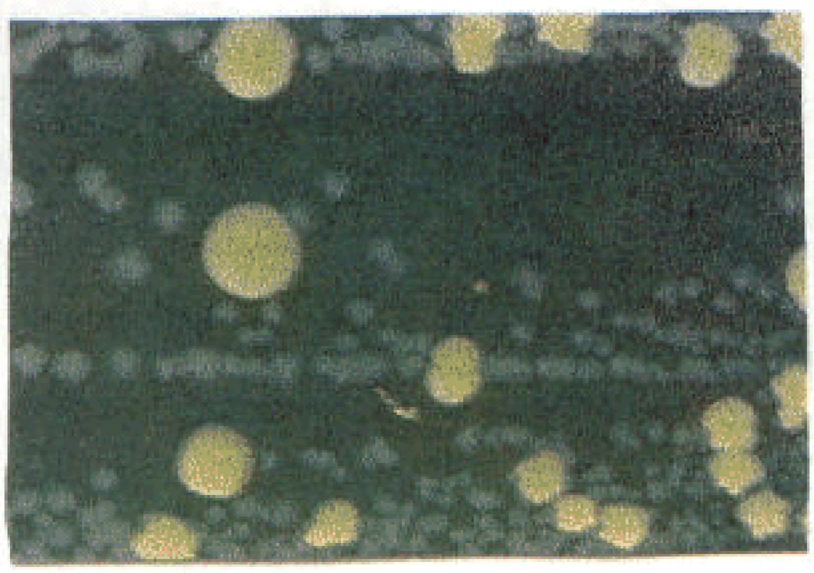
Gram positive L. monocytogenes.



On blood agar, tiny pinpoint colonies with small zones of beta hemolysis.



Colonies are transclucent, bluish-grey and 1.5 mm in 24 hours. The large opaque colonies are S. aureus.



After intraperitoneal injection into a mouse, from a 24 hour broth culture, necrotic foci are seen on the liver. Larger and more numerous lesions are seen on the spleen 4 days after infection.



Toxicity and Harm

There are four major serological groups based on their O and H antigens. Type 1b accounts for most of the disease causing strains recovered.

Tests for pathogenicity is performed by adding a drop of a 24 hour broth culture into the conjunctival sac of a young rabbit or guinea pig. The opposite eye will serve as an uninoculated control. L. monocytogenes produces a severe purulent conjunctivitis within 24-36 hours. It also produces monocytosis when injected into the blood.

It is not toxic to rats and pigeons and in chick embryos will produce focal lesions of the chorio-allantoic membrane.

Klebsiella Pneumonia

History and Recovery from Nature

K. pneumonia causes severe enteritis in children and causes pneumonia and other respiratory tract infections in man. It can also cause septicemia, peritonitis, and meningitis.

Some texts separate Klebsiella into three species - K. pneumonia, K. rhinoscleromatis which produces nodules in the nose, and K. ozenea which causes upper respiratory tract and smelling disorders. Most texts only address K. pneumonia and differentiate them on the basis of their capsular antigens. More than seventy types have been identified and require the use of capsular antisera in typing procedures to identify correctly. Some species are parasitic while other strains are saprophytic.

Most of the pathogenic forms of Klebsiella are K pneumonia and these are known as "Friendlanders" bacillus, as he was the first to describe them as an etiological agent of pneumonia. Those strains found in the upper respiratory tract are usually heavily encapsulated Other strains are widely distributed in nature and are found in soil and water as free living forms.

K. pneumonia are differentiated from other enterobacteriacaea easily because they ferment lactose, are methyl-red-negative, Voges-Proskauer-positive, and grow on citrate. They produce gas in lactose and sucrose and hydrolyze urea. They are differentiated from proteus strains because they do not deaminate phenylamine. K pneumonia is differentiated from other Klebsiella by being urease and malonate positive while other species are negative.

K. pneumonia is responsible for about 1% of bacterial pneumonias. It is important as a weapon because it produces very high fatality rates of up to 50% even when antibiotics are used. These strains are serotypes 1 and 2. Most of the outbreaks occur in men with alcoholism, diabetes, or other predisposing conditions and infections. A thick, nonputrid, bloody sputum is produced in 25-75% of the cases. K. pneumonia causes abscesses and necrosis to a greater extent than most other organisms in the lung infections.

The majority of Klebsiella are isolated from water supply samples where they are saprophytic. The commonest infection in man is the urinary tract from which they are recovered and isolated. About 5% of the strains are recovered from the intestinal tract of healthy humans where they exist as commensals in about 30% of the population. This makes it the second most common enterobacteria found in the human intestine behind E. coli.

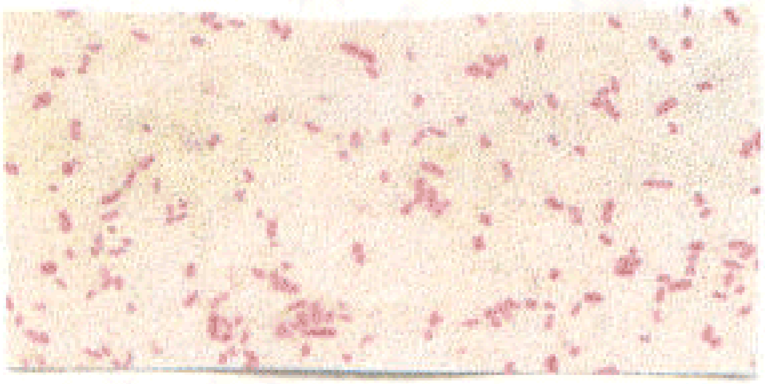
The normal intestinal flora interfere with colonization by Klebsiella. When antibiotics are used, the flora are reduced allowing colonization by resistant Klebsiella strains and this is commonly observed in hospitals where the number of K. pneumonia organisms is found to be much higher.

In hospitals, serious epidemics have occurred among newborns and endemic infections are seen in urological wards.

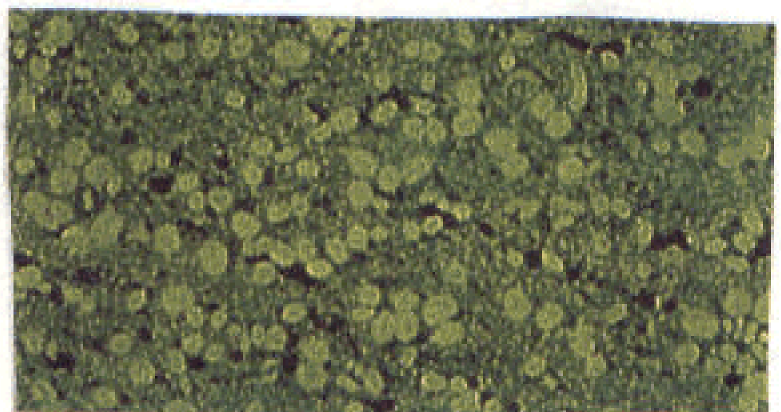
Growth and Production

Under the microscope, Klebsiella appear as short to long rods from .5-1 *u*m in width and 1-4 *u*m in length. They are all non-motile, non sporing, stain uniformly gram negative and all produce capsules. Most strains have fimbriae which act as adhesins and are virulence factors.

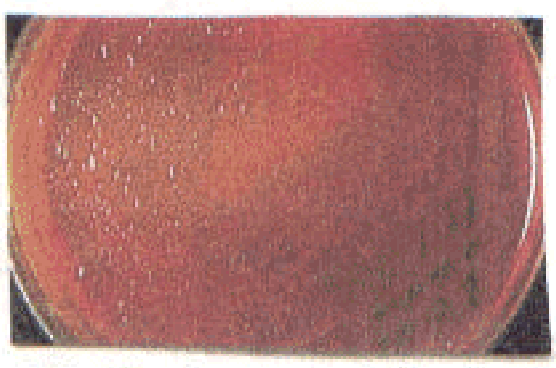
Friendlanders bacilli appear almost oval under the microscope. They occur singly and in pairs.



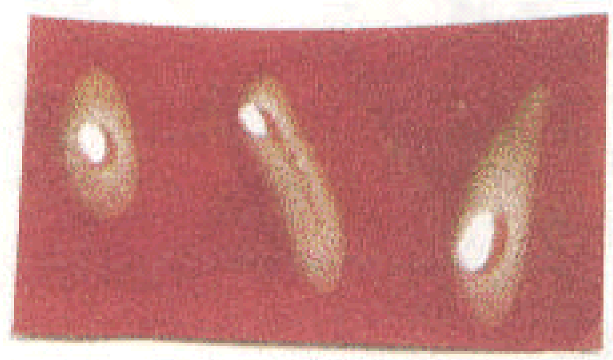
A smear of Klebsiella mixed with India Ink making it easy to see the halo (capsule) surrounding each cell.



Klebsiella growing on blood agar at 18 hours. Notice the mucoid character of the heavy growth.



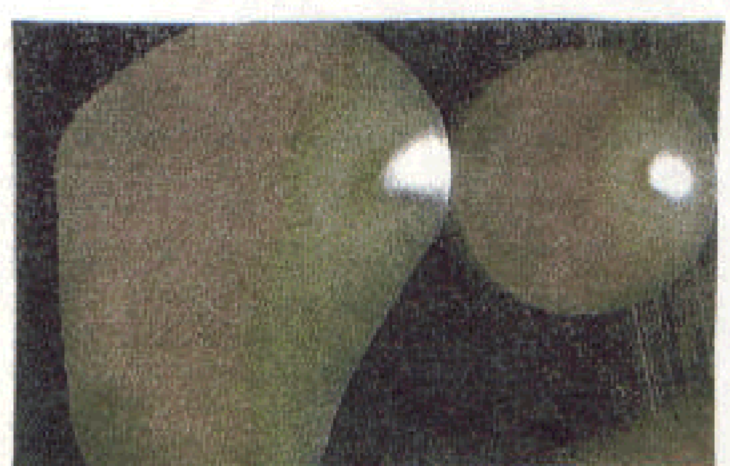
Close up of the individual colonies that are large, raised and viscid.



On MacConkey agar the isolated colonies are large, domed, and in this case, smooth and viscid. They are pink instead of the deep red of E. coli.



Large colonies on MacConkey agar due to greater supply of lactose which Klebsiella use to make more extracellular polysaccharide.



Klebsiella cultures appear almost identical to E. coli in which the colonies are large, and raised, but on MacConkey agar the colonies appear light pink rather than red due to the fermentation of lactose. Most of the strains are viscid to mucoid rather than butyrous and this is due the large amounts of polysaccharide capsular material which we see as an abundance of extracellular slime. Most of the strains produce a distinctive yeasty odor.

Toxicity and Harm

Most smooth forms possess O antigens while non-mucoid smooth forms possess a K antigen which appear as the capsule. Non capsulate mucoid forms have an M antigen and mucoid and capsulate forms possess both an M and K antigen. Rough colony forms have R antigens and may also have M and/or K antigens. The M and heat stable K antigens mask the presence of O and R antigens.

Virulence is usually not associated with capsular types but the most infective strains in humans tend to be the lowest serotype numbers. The most virulent and invasive strains have mannose sensitive pili which serve as adhesins which permit colonization of mucosal surfaces. These pili also inhibit phagocytosis and impair intraphagocytic killing.

It is also known that some diseases and physiological states somehow increase the number of receptor sites that Klebsiella can adhere to on epithelial cells.

Some of the K. pneumonia produce a heat stable enterotoxin similar to that of the E. coli ST and LT toxins and some strains also produce sidephores that behave as aggressins and compete for available iron.

When invading lung tissues in pneumonia, they eventually cause disintegration of the alveolar wall and necrosis of the lung parynchema.

Protective Measures

Klebsiella are penicillin resistant but have responded to sulfonamides and tetracyclines. Plasmid mediated drug resistant has enabled modern K. pneumonia strains to become resistant to most antibiotics. In fact, more than 90% of the strains are resistant to at least 10 antibiotics each. In these case the only commonly used antibiotic that has been effective is Gentamycin which the author used successfully in swine herd operations in which Klebsiella was recovered. In these circumstances, Klebsiella was never the only infective organism.

Cephalosprins have also shown some effectiveness in treating Klebsiella and can also be used to differentiate Klebsiella from the genus Enterobacter.

Incorporation into Weapons

K. pneumonia should be distinguished by chemical tests and lower serotype numbers used as the primary strain. It is more invasive when combined with other disease causing organisms and should be used in combination weapons only.

K. pneumonia should be used as a primary inhalation weapon. It has achieved up to 90% mortality rates in humans in untreated cases when it successfully infects and causes pneumonia. It can also be used in piercing weapons where it can easily cause infections under the skin and spread to other body tissues.

The most effective strains are those obtained from hospitals where most of the organisms already show strong and widespread antibiotic resistance.

Proteus

History and Recovery from Nature

There are five species of proteus that are recognized in nature as causing disease. They are second only to E. coli in causing urinary tract infections and are more destructive. Because of their urease activity they are particularly destructive to the kidneys. Proteus are the easiest enterobacteriacaea to identify because they are the only ones that deaminate phenylalanine. Four of the five species produce urease within 4-6 hours. This enzyme hydrolyzes urea to ammonia and carbon dioxide at a rapid rate which causes various injuries.

Proteus is an opportunistic pathogen and most infections are hospital associated.

Proteus are found in soil, water, sewage, decaying animal matter-especially in water, on garden vegetables and in human and animal intestinal tracts. They are often recovered following bowel infections. They are motile at 37 C due to numerous lateral flagella and produce a thin translucent sheet of growth on most agars and this action is referred to as "swarming".

Proteus species are aerobic and facultatively anaerobic. Most strains are fimbriate, non sporing and non encapsulated. They grow well on all routine media at 37 C and most will rapidly swarm over the entire surface of moist agar plates. This means that discreet colonies will only exist during the first few hours before spreading and running together. Proteus has a strong seminal odor which is hard to miss and this can aid in screening and recovery of the organism.

They also are the only enteric bacteria to grow in alkaline pH which allows for easy differentiation of the species.

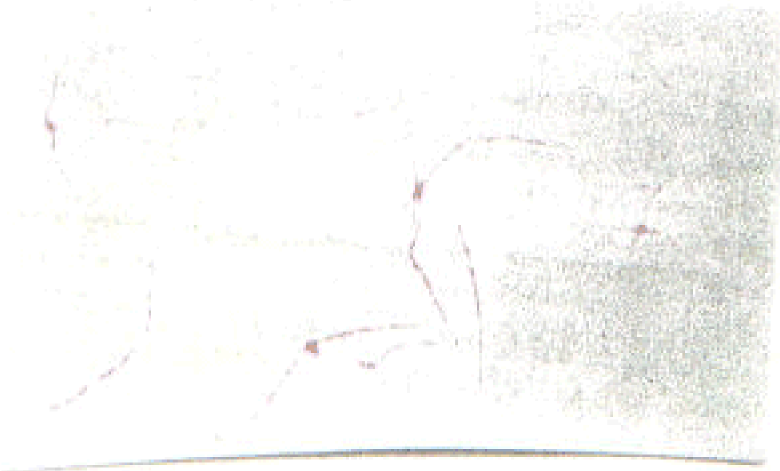
In addition to being a significant cause of urinary tract infections, Proteus can also cause wound infections, pneumonia, and septicemia demonstrating its ability to invade and grow in human tissues. The pneumonia behaves in a similar manner as the Klebsiella pneumonia and has a similar high mortality rate in respiratory and blood related infections.

Growth and production

When proteus swarm over the entire culture plate, the culture appears as a thin film which is often missed by careless lab technicians. Swarming can be prevented by increasing the agar or gelatin to 5%, or by adding .01-.04% tellurite or .25% chloral hydrate, or .025% sodium azide to the media. DCA will also inhibit swarming. The swarming occurs in concentric waves which appears as a series of rings originating from the center of growth which makes it easy to differentiate and isolate.

Proteus are generally rod shaped, 1.5 *u*m x 3-5 *u*m with pleomorphic forms being quite common, especially in young swarming cultures. Curved or filament forms may reach up to 30 *u*m in length. These long and slender forms give way to short forms once the entire surface of the culture media has been covered. They are nonencapsulated and stain uniformly gram-negative.

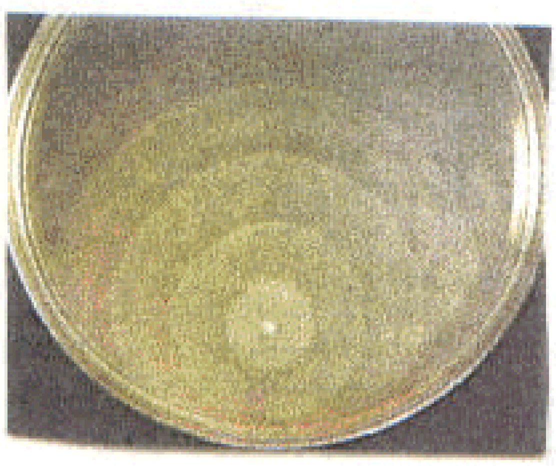
Protues from swarming growth
at 7 hours and 37 C.



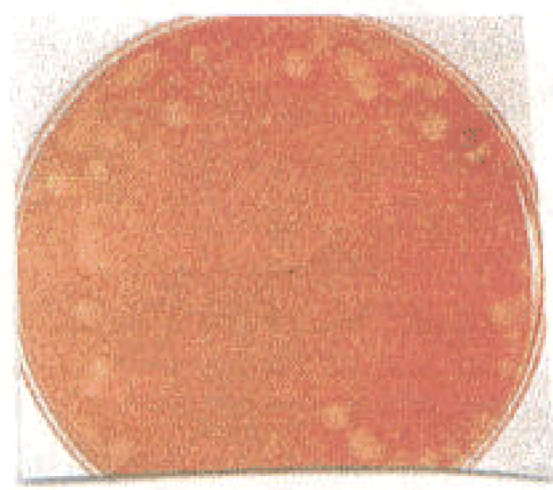
After 48 hours with swarming stopped
the cells look typical for enteric bacteria.



Swarming in concentric waves with
contour lines evident from around the
point of inoculaion. On moist media
the organism may swarm without stopping.



A way to stop swarming is to incubate
the media with the organism and then
add a layer of gelatin or agar on top of it.
This inhibits proteus as shown.



All proteus ferment glucose but do not act on lactose. Two of the species liquefy gelatin and most produce gas. Anaerobic growth is usually poor and almost all isolation and growth is done aerobically.

<u>Toxicity and Harm</u>

Proteus are often recovered from the stools of individuals receiving antibiotic therapy, particularly, acute cases of dysentery, and have also been recovered from the blood.

All the proteus species produce O antigens and most have H antigens like the other motile enterobacteriacaea, which are used in serotyping. More than 200 serotypes are known.

Infections of the eye, ear, peritonitis, and suppurative abscesses at various sites are among the infections observed with Proteus species. It is also a major contributor to infant diarrhea.

Protective Measures

Streptomycin and tetracycline's have been the most effective antibiotics but resistant strains appear to be on the increase. Aminoglycoside antibiotics are the best overall choice in modern drug therapy as well as combinations of trimethoprim and sulfamethoxazole.

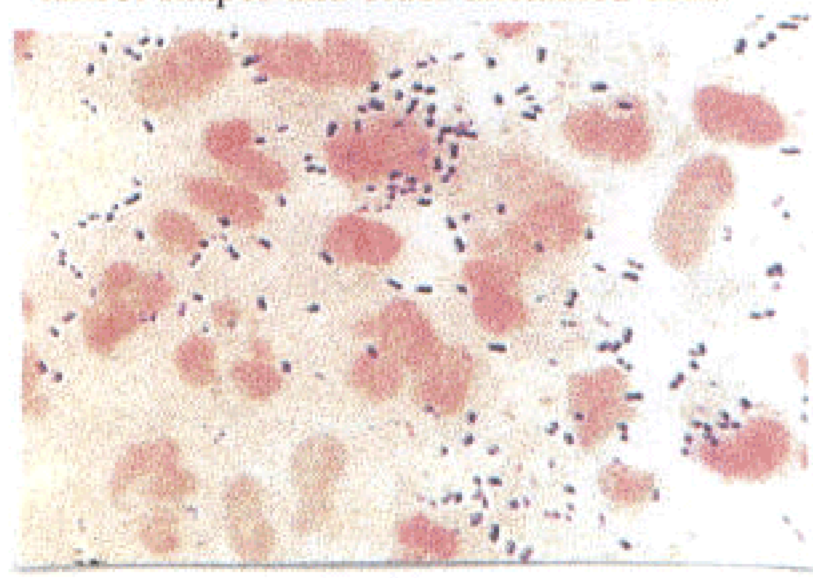
Incorporation into Weapons

By itself, Proteus is usually not an effective weapon unless septicemic conditions are involved. It is best used in combination with other organisms and toxins in "cocktail" weapons.

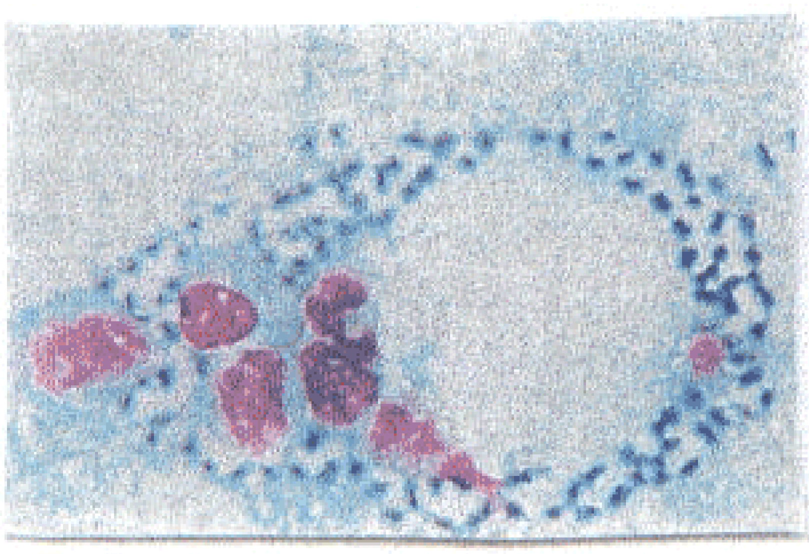
Short chains and pairs of pneumococcus. A typical smear showing the many forms.



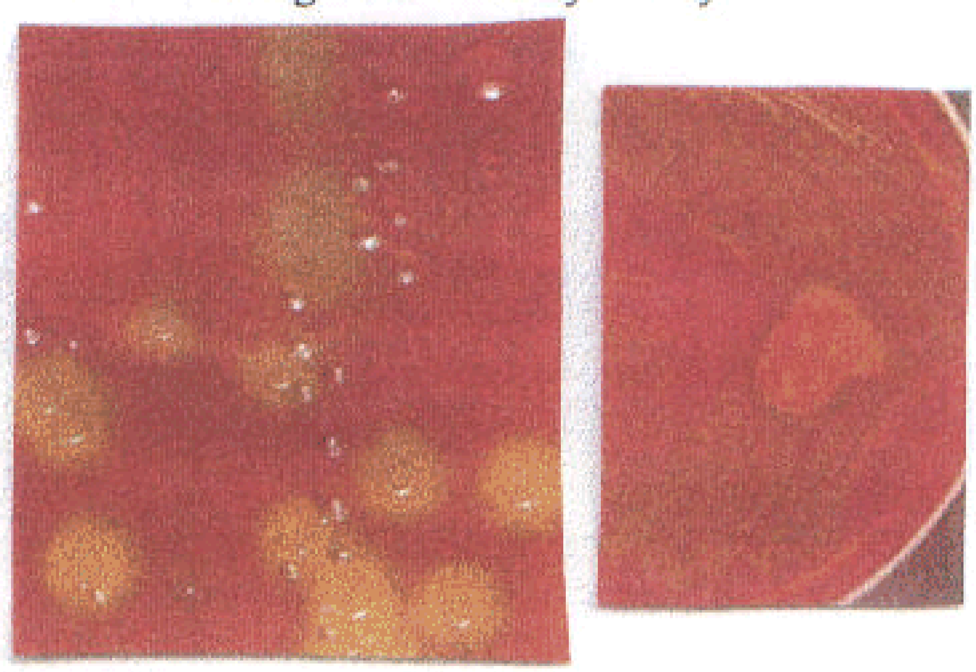
Many pairs of pneumococci in cerebrospinal fluid. Note the lancet shapes and older unstained cells.



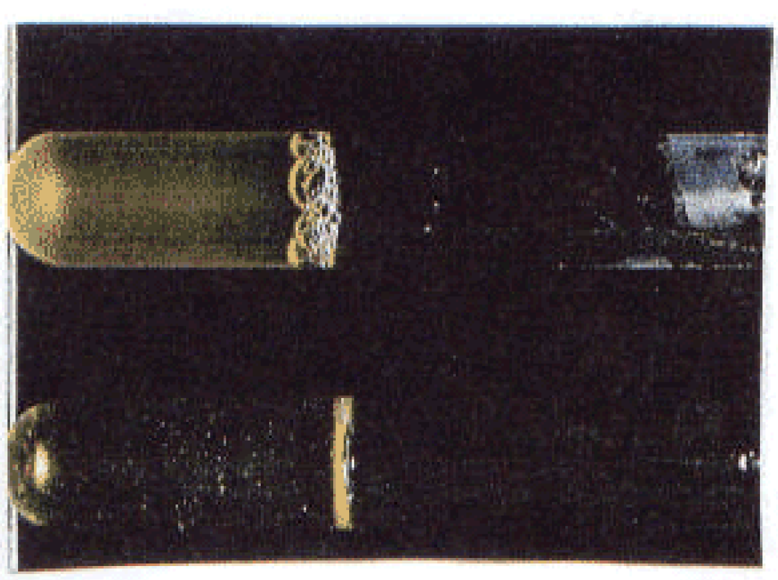
Blue stained pneumococcus capsules with the red-blue cells in the center. Taken from the peritoneum of a mouse whose cells are seen as large and red.



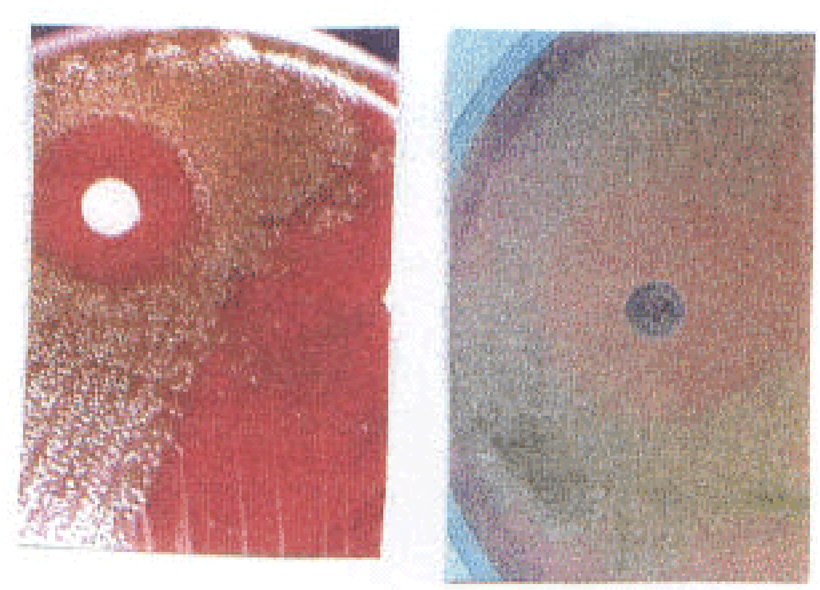
Two drops of 2% bile were added to these plate of S. pneumonia culture. The colonies dissolved in 30 minutes as shown leaving behind hemolysis only.



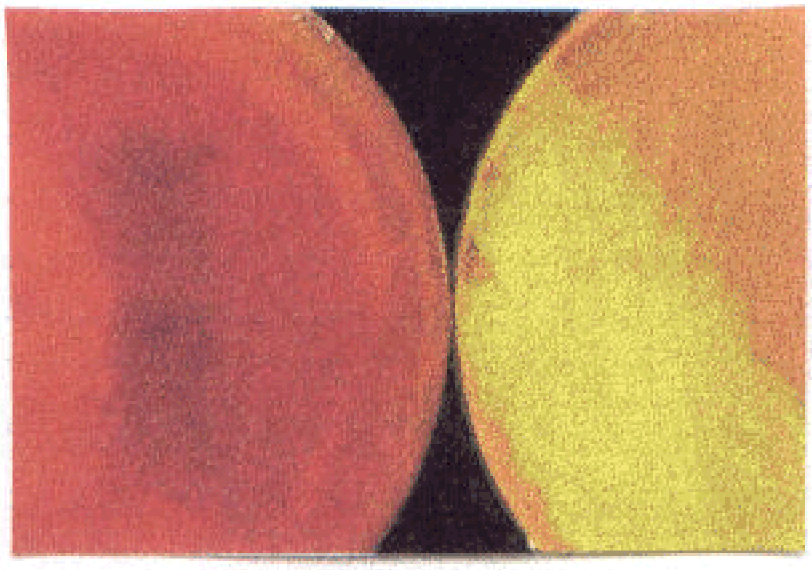
Test tube bile solubility test. The bottom tube has bile added to the culture and has cleared showing that the S. pneumonia cells have dissolved.



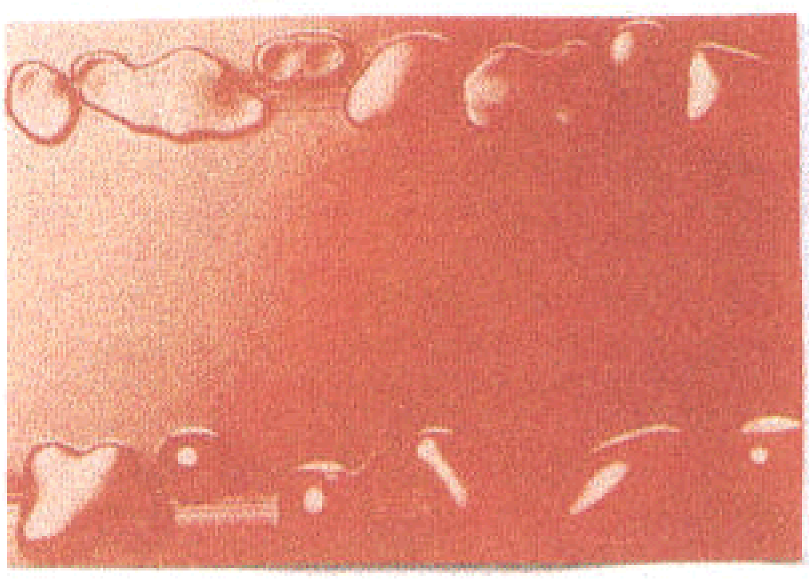
Two types of S. pneumonia on plates with optichin discs. On the left are mucoid colonies, on the right, non mucoid with clear alpha hemolysis The optichin inhibits growth.



Two plates- the left is ordinary blood agar-the right has heated and broken down blood in which the greening produced by pneumococci is easily seen. The intact red blood cell is not needed for alpha hemolysis as it is for beta hemolysis.

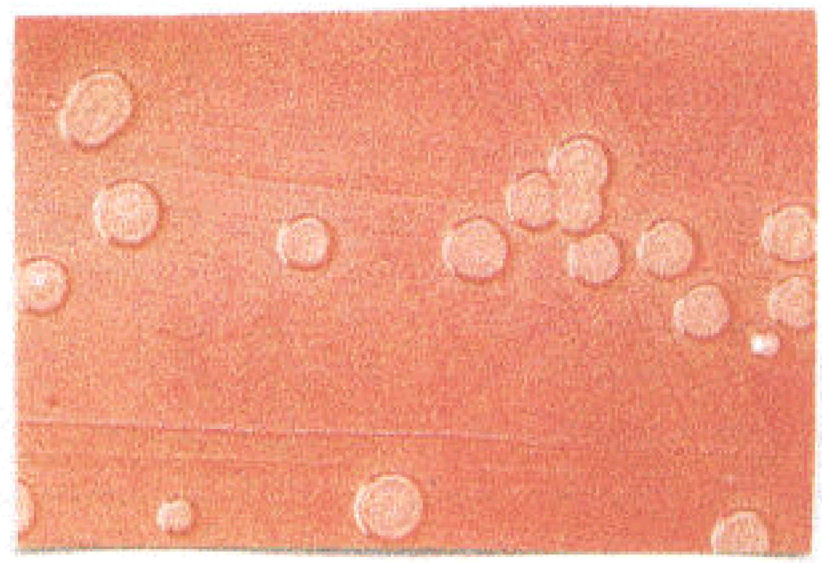


Mucoid colonies at 18 hours on blood agar. Isolated colonies are large, moist and convex and can be seen starting to run together.

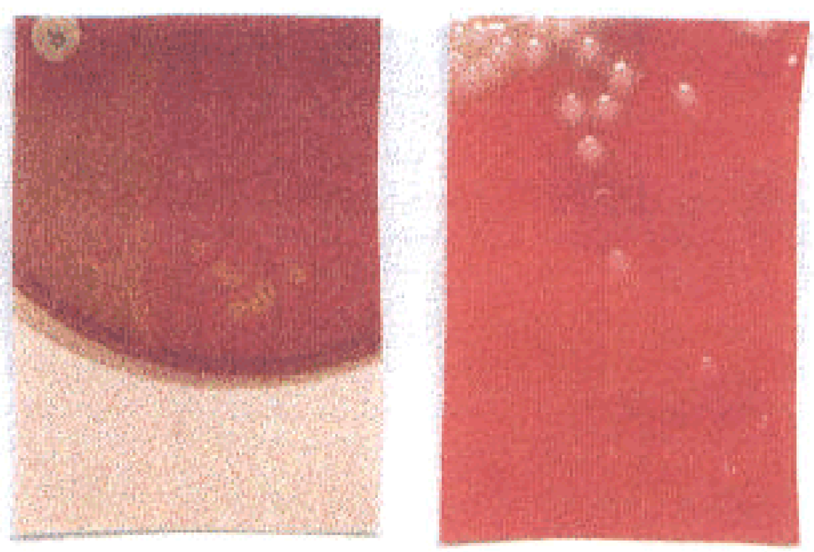


"Draughtsman" colonies of pneumococcus which are flat with raised margins.

Sometimes concentric rings are seen which give it the name "draughtsmen".



Rough colony growth on the left- Smooth colony growth on the right.



Glucose and glycerine added to the culture media speeds the rate of growth but also speeds up autolysis by increasing production of acid end products. Increasing CO2 content (5%) of the medium or air increases growth rate and isolation from other contaminating organisms as well. [Increasing CO2 can be easily accomplished by simply burning a candle in the incubation container such as a jar until the loss of oxygen causes it to extinguish the flame.]

If peptone is added to the medium, it should be added before the medium is heated to reduce factors in the peptone that might inhibit growth of the colonies. Metallic impurities in various media may also inhibit growth.

Pneumococci
(Streptococcus pneumoniae)

History and Recovery From Nature

Pneumococci have been reclassified as part of the Streptococcus Genus and is technically called S. pneumoniae because of its similar biological and chemical characteristics with other members of the streptococci. Its adoption as part of this genus was also due to reconciling the classification with European scientists. For purposes of studying its disease causing abilities and potential use in biological ordnance we will treat it as a separate entity here.

Pneumococci are part of the normal flora in the upper respiratory tract of many mammals including man. They are usually present in most pneumonia but are not always recovered from sputum or lower respiratory secretions because of overgrowth of antagonistic organisms in the pharynx and problems with culturing. Man and a few animals are the only natural reservoir for pneumococci.

The pneumococci were first recovered and studied by Pasteur in 1891 and by Stenberg in the United States. Its role in pneumonia was not recognized for several years however. The study of transformation in pneumococci in the early part of the 20th century led to the eventual discovery of DNA.

Infants are colonized within hours of birth and the carriage rates for the very young is very high worldwide. About 1/3rd of all preschool children are colonized with various strains of pneumococci. The rate gradually declines into adulthood reaching a low of about 10% and then increases again at about age 55. Children with sickle cell disease are especially susceptible to infection when they acquire the organism from a healthy carrier.

Methods of increasing recovery of pneumococci from sputum involve inoculating mice with the sputum sample, adding gentamycin to culture plates at 5 um/ml to inhibit antagonistic bacteria, and transtrachea aspiration to recover organisms from deeper tissues.

Pneumococci cause infections in the lungs, meninges lining the spinal cord and brain, endocardium and other tissue sites, usually after other microorganisms have made the host susceptible. The elderly and alcoholics have been especially sensitive and prior to the advent of antibiotics it was known as the "old mans friend". Pneumonia is the only infectious disease that is near the top of the common causes of death in man in the United States each year. Mortality is about 30% in untreated and 5-10% in treated cases. The fatality rates go up as the pneumococci invade the bloodstream with resulting bacteremia.

Pneumococci are very sensitive to penicillin derivatives which have made the rapid curing of pneumococcus infections possible when diagnosed early.

It is recovered from sputum, joint fluid, cerebrospinal fluid, and other body secretions in both healthy and affected individuals. It has been recovered and cultured from dried sputum samples after several months but seldom survives in artificial culture mediums for more than a few days. It causes infection due to the high virulence in various lung tissues causing pneumonia which gives it its name.

Colonies first form on blood agar aerobically at 18-24 hours after incubation. The virulent strains that produce capsules and make the most affective weapons appear as moist, glistening, mucoid, transparent, dome shaped, circular or oval colonies that tend to run together. The poorly encapsulated strains that are not as deadly produce small, round, translucent colonies that start out as convex but develop a central depression due to autolysis as the core cells die and decompose. The addition of the capsule makes the more virulent cells produce the larger colonies. All types produce a 2-3 mm zone of alpha hemolysis.

In anaerobic incubation, the colonies are surrounded by a zone of beta hemolysis which is due to an oxygen-labile pneumolysin O that is not produced in the aerobically grown colonies. In artificial cultures, their is an absolute requirement for choline as part of the growth medium which differentiates the pneumococci from other streptococci.

Bile salts, particularly the sodium deoxycholate and sodium taurocholate have the ability to selectively lyse (kill and decompose) S pneumonia. By adding these salts (dry or liquid-5-10% in liquid) to actively growing colonies on a culture plate or test tube, the cells dissolve in 15 minutes leaving only the background hemolytic effect that is seen. The S. pneumonia produces a self lysing enzyme called amidase that accounts for the depression seen in older colonies of the less virulent strains. This effect is accelerated in the presence of the bile salts which is why the S pneumonia does not colonize the intestinal tract like other strep may do. Other streptococci do not produce this autolyzing enzyme and are not affected by the bile salts.

If bile salts are not available, other surface active agents like sodium lauryl sulfate (found in hair shampoo and laundry detergents) and other similar detergents also cause the dissolving of the pneumococcal cells by activating the autolysis enzymes.

Pneumococci are extremely sensitive to optichin (ethyl hydrocuprein hydrochloride). Small paper discs are impregnated with a drop of solution containing 1 part optichin in 4000 parts water. These discs can be placed on the culture media or the liquid can be streaked on the plate. The pneumococci are inhibited from growing within 5-15 mm of the treated area. Other Strep species will grow near or directly up to the optichin which allows for easy separation.

Intraperitoneal (the two layered membrane that lines the wall of the abdominal cavity and covers the abdominal organs) injection in mice of sputum samples allows for recovery and growth of the most virulent pneumococci organisms from the resulting infected tissue growth. The test mouse tissues are very sensitive to the capsular types of pneumococci and this results in a fatal infection. The mouse usually dies in 16-48 hours.

A single cell injected into the intraperitoneal tissues is sometimes sufficient to produce a fatal infection. The mouse tissues usually eliminates all other injected organisms making it easy to recover a pure culture of the virulent strain. Large numbers of the pneumococci cells are recovered from the heart blood or from the peritoneal exudates with microscopy showing large numbers of encapsulated diplococci.

Growth and Production

Stained pneumococci appear as gram positive diplococci with the pairs of diplococci app. 1 μm in diameter and tapered at one end in the shape of two lancets with the opposite sides flattened.. If the pneumococci are found to have capsules, this is an indication of virulence and these strains make the most effective weapons. When stained with India ink, the capsules take the form of easily seen halo's on slide preparations. Unstained halos can sometimes be seen in the gram stains.

Pneumococcus are non-motile and do not form spores. In exudates, its well defined capsule envelopes the diplococci but this is usually not seen in cultures unless blood or serum are added.

There are 82 (as of 1989) serotypes of pneumococci all of which have commercial antiserums available. Older cultures die off due to sensitivity to their own metabolic byproducts and this causes the older cells to stain gram negative and autolyse over time.

The colonies have plateau surface shapes that later develop elevated margins and concentric ridges due to aging of the colonies and autolysis of the older cells. This effect produces the "draughtsman" colonies. Continued laboratory subculture results in longer chains of cells observed under the microscope due to cell wall adhesion during division.

The pneumococci form short chains and are more rounded in broth cultures and encapsulation is usually not evident in the liquid mediums. Cultures older than 48 hours are often swollen and irregular and often lose their gram-positivity.

Pneumococci grows both aerobically and anaerobically in a temperature range of 25-40 C and a pH range of 6.5 to 8.3. They grow on all ordinary media but best on blood and enriched media (heated blood).

S. pneumonia is sensitive to pH changes growing best at 7.6-7.8 and at 37 C. The colonies self lyse in the presence of surface active agents like bile which can be used in identification and autolyse in older cultures producing the small craters (crateriform) effect. They are often surrounded by a greenish halo on blood agar from the alpha hemolysis. This green pigmentation is more obvious when growing on heated blood agar.

In aerobic cultures, hydrogen peroxide is produced along with acetic and formic acids. It does not produce catalase or peroxidase enzymes so the accumulation of these materials kills the organisms as the culture ages. This effect can be postponed by the addition of catalase to the culture medium. Erythrocytes are an excellent source of this enzyme and adding it to the medium (and possibly to biological ordnance) will aid in colony survival.

In order to maintain colonies for use in weapons continuos subcultures on blood agar is required every few days or the colonies may be rapidly vacuum dried for long term storage and use in weapons. Repeated subculture causes a loss of capsulization and virulence which is seen by colonies changing from smooth to rough forms and serotype changes. This is not recommended if the organisms are to be used in ordnance. The capsules are most easily seen in wet mounts of the organism using India ink.

It is also possible to preserve some strains in storage by refrigeration of the cultures or by lyophilization..

The addition of heart tissue or infusion in agars or broths enriches and aids the production of organisms (which is why the organisms are so readily recovered from the heart blood on autopsy in infected mice).

Toxicity and Harm

The most common human infection produced by S. pneumonia is lobar pneumonia. It causes fever, chills, and sudden pain in the chest. The sputum is a characteristic rusty color due to the bloody exudate that is produced. Bacteremia is common (25%) in the early stages and can develop into sinusitis, mastoiditis, and meningitis. The organism is present in huge numbers and are easily recovered from the sputum. If the organism reaches the lung parenchyma by inhalation or peribronchial lymphatics, it may spread to the bloodstream resulting in many complications such as meningitis, endocarditis, etc.

The lobar pneumonia is a communicable infection which means that it can be induced in large population by means of inhalation aerosol and dust weapons. The capsular strains have special invasive properties that allow for easy creation of natural epidemics in semi-closed communities like military barracks, factories, schools, etc. Carriers recovering from pneumonia may harbor the infective organisms for a long time and spread them via the sinus drainage and in coughs or sneezes. Contact carriers resistant to infection may carry the organisms and spread the infection themselves.

Predisposing factors must occur before the organisms are able to initiate infection. These include injury to the respiratory tract such as caused by viral infections, excessive smoking, silicoses from mining, circulatory and respiratory defects as a result of obstruction or congestion, and general conditions like malnutrition, fatigue or general debility.

In pneumonia infections, the pneumococci invade alveolar tissues which results in outpouring of edema fluid. This allows for rapid multiplication and spread of the organisms to other alveoli. Leukocytes and red blood cells accumulate in the affected alveoli leading to complete consolidation of the lobe or segment. This crowding in the alveoli leads to phagocytosis and the resulting end of the infection as the invading cells are very rapidly ingested and destroyed by the phagocytes. Only the strains with capsules are able to avoid this type of human host resistance.

In some areas of the country, more than 50% of the population carry the virulent capsular forms of the pneumococci in their nasopharynx allowing for easy recovery and production of the organism. The thick mucoid portions of the sputum rather than the watery saliva portions most often contain the virulent pneumococci.

The pneumococci are responsible for causing bacterial pneumonia in one out of every five hundred people in the United States each year. The healthy contact carriers are the largest reservoir of the disease which occurs in epidemic outbreaks during the winter months following the spread of the annual flu viruses which modify the tissues and weaken the host defenses.

The pneumococcus can damage the tissues of the host (target) as long as it is able to remain outside the defensive phagocytic cells. It protects itself from phagocytosis by producing a capsule which exerts a antiphagocytic effect. The mouse test described earlier produces death in a few hours to days with the capsular strains while it is able to resist invasion by non-encapsulated strains. This allows for recovery of the most virulent potential weapons. When the colony is treated with an enzyme that removes the capsule, the strain is rendered nearly non-virulent and renders it susceptible to phagocytosis.

It has been found that large amounts of free capsular material is present in the bloodstream, a high mortality rate of the patients is produced. The capsular material appears to neutralize the specific antibody to the pneumococci (other antibodies ?) which makes it inaccessible to stop the invading organisms. The use of large volumes of capsular organisms enhance the ability of the pneumococci to infect and maintain itself in a host while producing high mortality rates.

Pneumococci also produce **Pneumolysin O** which has properties similar to that of the streptolysin O already described. It is oxygen sensitive hemolysin that is produced only under anaerobic growth which occurs in the presence of excessive mucous and other exudates. It is a lethal, dermonecrotic and dermotoxic, cholesterol sensitive, SH activated cyclotoxic toxin. It is water soluble and filterable and accounts for the alpha hemolysis seen on blood agar.

Neuraminidase is a glycosidic enzyme that is produced by fresh colonies of S. pneumonia. As already mentioned, the neuraminidases are produced by diphtheria and myxoviruses. They act on glycoprotein and glycolipid substrates in cell membranes and body fluids where they break down the cellular substance into material that can be used as food for the invading microorganisms (such as mucus). This enzyme and others similarly produced allow the pneumococci to live on the mucus membranes of the human nasopharynx and bronchial tree.

Leucocidin is also produced along with a separate necrotizing substance similar to the one formed by some of the staphylococci. Many strains also produce hyaluronidase, especially when hyaluronic acid is added to the growth medium (adding hyaluronic acid as an enhancement is a potentially very effective method of increasing virulence). A purpura producing substance has also been identified that produces a discoloration of the skin in albino mice.

Currently it is believed that pneumococci produce disease mainly from their ability to invade and multiply in many human tissues. There have been observed biochemical alterations in human body tissues that cannot be accounted for in those produce in culture media. It is surmised that a toxin is produced only in host tissues that allows the pneumococci to cause harm that has not yet been isolated or defined.

It is known that S. pneumonia physically adheres to the pharyngeal cells accounting for its high carriage rate among healthy individuals. The most virulent strains are able to live in and on mucus membranes through the production of an immunoglobin protease that helps break down the mucus into substrate. As the disease progresses, the cells penetrate into the tissues from their colonization sites in the mucus membranes by factors separate from the toxins produced and still unknown to researchers.

It is also known that, like the other streptococci, the pneumococci cause an immune response that causes the body tissues to harm themselves. Part of the theory centers around the action of phagocyte cells and the production of histamine and serotonin that combine to cause inflammatory tissue injury. The additional release of enzymes and toxic oxygen metabolites from neutrophils also occurs while they are ingesting the pneumococci which may also cause injury. In combinations of these actions, the host cells may harm itself while attacking the infection.

Animal testing for virulent strains involves injecting rabbits or mice subcutaneously with a young culture. This produces a rapidly developing septicemia and death in one to three days. At autopsy, typical capsulated diplococci are recovered in large numbers from the heart blood and are the best cells for culture in weapons development. The virulent strains must be grown on blood to avoid loss of virulence. Subculture should be avoided where possible and rapid vacuum drying of sputum, cultures, or other fluids should be done to preserve the effective strains.

When combined with other invasive or injury causing toxins and organisms, the pneumococci are capable of producing very high fatality rates among all target populations.

Use of irritants that produce mucous aid in establishing infection and allowing the pneumococci to anaerobically grow and produce pneumolysin O while reproducing under the protective exudate. Any type of chemical, toxin, or material that causes accumulation of fluid in the alveoli of the lungs will assist in initiation of the infections.

Using a blood clotting agent to prevent a defensive response and stop antiserum or antibodies from reaching the infected sites will aid in overcoming vaccines, antiserum, and other host or manmade defenses.

Highest mortality rates in hospital patients involves infection with numerous strains of S. pneumonia. This commends the production and use of multiple strains in bacteria based ordnance.

This particular organism lends itself well to disguised and clandestine attack in winter when it would be practically indistinguishable from the expected background epidemics.

Salmonella

History and Recovery from Nature

There are 14+ known species of Salmonella that are recognized as disease causing agents. These can be further subdivided into more than 1600 known serotypes.

Typhoid is probably the best known of the diseases caused by Salmonella. The most famous is the case of "Typhoid Mary", Mary Mallon who acted as a carrier for the organism and passed it on to 26 members of seven different families.

After about one week from exposure to typhoid, the patients become seriously ill with fever sustained at 104 F and acting delirious. The abdomen is very tender and may have rose colored spots and diarrhea begins in most patients. By the third week, patients are exhausted and febrile but begin to improve if no complications have set in. Mortality is 2-10% with relapse in about 10% of the cases. About 3% become chronic carriers (mostly women) who serve as sources for future infections. The gall bladders are the primary reservoir.

S. typhi, the cause of typhoid fever is the most potent disease causing strain and most other salmonella species share its same biochemical characteristics. Most other species have invasive differences which make them similar in nature to S. enteritidis.

S. typhi are aerobic but facultatively anaerobic. They are non-sporing and grow on routine media between 6 and 8 pH and from 15-41 C. Their optimal growth temperature is 37 C. At 24 hours their colonies are smaller and more delicate than E. coli. Colonies on blood agar are large, thick, greyish white, moist, circular discs that may be dome shaped and smooth or rough. The opacity and size vary with the strain. Rough colonies may be irregular, dull, effuse, and dry. On MacConkey agar they will appear pale or colorless.

Adding sodium tetrathionate and selenite improves the growth and selective culture of the Salmonella species. Most other enterobacteriacaea cannot use the reduction products of these substances while Salmonella can thus allowing favored growth. Adding desoxycholate will make the media selective for the Salmonella species because they can tolerate and reproduce in a large amount of bile while many other organisms cannot.

Selective cultures and enrichment growth techniques have already been described in the enterobacteriacaea section and will not be repeated here.

S. typhi produces acid and no gas from dextrose, maltose, mannitol, dextrin, and trehalose by fermentation. It will not ferment lactose or sucrose and does not decarboxylate lysine. S. typhi survives for months in moist cultures and ice making it easy to store without subculture. It survives in sewage and tap water for weeks. It is killed at 56 C in one hour and is usually destroyed by either chlorination or pasteurization or drying out. It survives in soil for months making it easy to recover from livestock yards. Cultures can be stored and remain viable for several months.

S. typhi causes typhoid fever. The infection is started by ingestion of the organism and incubation usually takes about 14 days but can be 3-21 days. It migrates from the intestines using the lymphatic system to reach the mesenteric glands where they may use the thoracic duct to invade the circulatory system. The liver, gallbladder, spleen, kidney, and bone marrow usually become infected during this bacteremic phase. This first phase takes about 7-10 days.

The S. typhi then invades the intestines from the gall bladder mainly affecting the lymphoid tissues (the flat, whitish lymphatic follicles in the mucosal and submucosal layers of the small intestine). Necrosis occurs with the characteristic typhoid ulcers. Hemorrhage and perforation of the tissues can complicate the conditions. The typhoid fever generally runs its course in several weeks.

S. enteritidis is by far the most common species of Salmonella with over 1400 serotypes having antigenic differences. Paratyphi A and B cause milder forms of typhoid fever called paratyphoid. All the other types are associated with enteritis or food poisoning. Most of these are similar to S. typhi in cultural and biochemical characteristics, although some are non-motile. Motility and pathogenicity are not related.

The disease caused by S. enteritidis are usually called salmonelloses. The enteritis is usually and inflammation in the small intestine causing the stools to become liquid. The infection usually begins about 18 hours after ingestion and lasts about 2-5 days. Enteric fever may develop with bacteria and/or toxins in the blood causing a rise in body temperature. This may proceed to meningitis, encephalitis, osteomyelitis, endocarditis, nephritis, or any combination of these. Most of the salmonella infections result in nasty enteritis of a short duration.

Salmonella also cause **pyemia**, in which localized infections and abscesses of different organs occur following the spread throughout the body of the organism. They can invade not only by the intestinal tract but via the pharynx and tonsils as well.

All Salmonella penetrate the epithelial lining of the small intestine but unlike other bacteria, they pass through this lining by an unknown biochemical mechanism to the subepithelial tissues. This process seems to be similar to phagocytosis whereby the Salmonella cause the brush border to degenerate and the bacteria enters the cell. They are then surrounded by inverted cytoplasmic membranes similar to the phagocytic vacuoles. After penetration, the organisms are ingested by macrophages where they multiply readily and may move on to other tissues in the body. The epithelial tissues are destroyed in the later stages of the disease.

Salmonella are considered to be intestinal parasites. They are found most often in sewage and from their usually contaminate the water supply and food. Human carriers may shed S. typhi bacilli for 2-12 months following initial infection while chronic carriers can yield the organism for much longer. This usually happens until they have surgery to remove their gall bladder.

Because of their wide distribution and easy spread, S. typhi and other Salmonella are difficult to control. Periodic flooding distributes the organisms contained in sewage to wide surface areas where it can become easily entrenched and endemic in vegetation and many animals.

Recovery of the organism is almost always from the sewage of humans or stool samples of animals. When the organism has spread, it can often be isolated from the urine in the 3rd to 6th weeks of infection. In third world countries where sewage flows into rivers, it can often be isolated in endemic areas simply from filtered water samples. In enteric fever, it can also be isolated from the blood. Rats, mice, pigs (S. cholera suis), chickens and other fowl almost always carry strains of Salmonella which can be reliably recovered from the intestinal tract or feces. It is almost always non pathogenic for the animals that they are recovered from and these are always healthy carriers for the organisms. The exceptions would be epidemic outbreaks of typhoid in canaries and turkeys.

S. enteritidis is widely distributed in fowl, swine, fish and clams and can almost always be recovered from intestinal samples from these species. S. arizonae can be isolated from the intestinal tracts of most reptiles and is sometimes associated with infections in man, dogs, and fowl, producing enteritis and enteric fever. [In 1970, more than 280,000 cases of salmonellosis were derived from pet turtles resulting in legal restrictions on the sale of the animals.]

In 1977, 27,850 cases of Salmonellosis were reported excluding typhoid fever in the U.S. Most infections are known to not be reported and health officials estimated the total at app. 2 million actual cases during that year. Poultry products are the largest source of infection in the United States. Dogs often harbor Salmonella for long periods with initial exposure from inadequately cooked dog food ingredients.

The main disease causing species are differentiated as follows -

	S. choler-suis	S. typhi	S. entertidis
Gas from glucose	+	-	+
Trehalose	-	+	+
Arabinose	-	-	+
Rhamnose	+	-	+
Ornithine decarboxylase	+	-	+
Citrate utilization	+/-	-	+

Salmonella produces two kinds of infections, enteric fevers and gastroenteritis. In both kinds of infection, the bacteria are ingested in food or drink and initiate the infection by adhering to and colonizing the intestinal tract. In the enteric fevers the bacteria invade the intestinal epithelium and then multiply in the intestinal lymph follicles and draining mesenteric lymph nodes. They then reach the bloodstream via the thoracic lymph. The Salmonella that cause this are the S. typhi and paratyphoid bacilli.

The gastroenteritis produces a superficial invasion that is limited to the epithelium where they multiply and produce enterotoxins. This causes the gastroenteritis symptoms and are known as Salmonella food poisoning. These are caused by the different S. enteritidis serotypes.

In acute gastroenteritis, the host suffers diarrhea, vomiting and some, especially children become septicemic. Typhoid, or enteric fever, causes bacteremia and tissue invasion in the first stage followed by the other symptoms described earlier.

A carrier state usually follows infection with discharge of the organism in large numbers for weeks following recovery.

Cockroaches and other insects can harbor Salmonella and act as reservoirs for infection for up to 21 days when fed Salmonella saturated food.

<u>Growth and Production</u>

Under the microscope, they appear as short plump rods, .5-.8 um wide and 1-3.5 um in length. They are actively motile using peritrichous flagella and stain uniformly gram negative. Their cellular morphology is indistinguishable from the other enterobacteriacaea without the use of selective media. They do not demonstrate capsules under the microscope although the Vi antigen (LPS) may be seen.

Most Salmonella possess the adhesive fimbriae that allow them to adhere to and establish themselves onto tissue surfaces. These are usually mannose sensitive types and are somewhat related to the virulence of the strain. The fimbriated S. enteritidis serotypes are much more virulent by the oral route than the unfimbriated strains. The production of enterotoxin appears to be a factor in aiding virulence which appears to assist the organism in invading the intestinal mucosa. This commends its use in combination with the organism in ordnance.

The colonies that are rough show a corresponding loss of virulence. When many colonies are grown, only the smoothest should be used in subculture. The colonies can be subcultured in mice to increase virulence (as seen in smooth colonies).

Most of the strains grow on simple synthetic mediums with ammonium salt as the nitrogen source and simple carbon nutrients like glucose, pyruvate and lactate. Most strains do not require vitamins or amino acids but some strains of S typhi need added tryptophan and including it in dust based ordnance aids in establishing infection.

Almost all strains will grow at room temperature making it easy to mass produce in enormous volumes in broth tanks which can also be used as delivery systems.

Toxicity and Harm

Most of the variations found in Salmonella species and serotypes are due to differences in their antigens. In culture you may see smooth motile and non-motile strains as well as rough motile and non- motile forms. The smooth cultures tend to be the most virulent and best at establishing infection and are the best to select for use in bio ordnance.

You can artificially increase both virulence and smoothness by repeated subculture in mice allowing for more effective weapons to be developed. The cause of the loss of virulence is due to the loss of O-specific side chains of the smooth LPS which leaves behind its basal R core and lipid A. The flagellar antigens are unchanged with the result that the colonies now appear smooth as the clusters of growing and dividing bacteria uniformly grow outward giving its smooth overall colony appearance. The O antigens are essential to protect the invading Salmonella from serum and phagocytic effects. The rough colonies that do not contain these antigens are easily attacked and destroyed by the bodies defenses.

When grown in broth, the smooth colonies produce a uniform turbidity while the rough colonies give rise to a heavy deposit and relatively clear supernate.

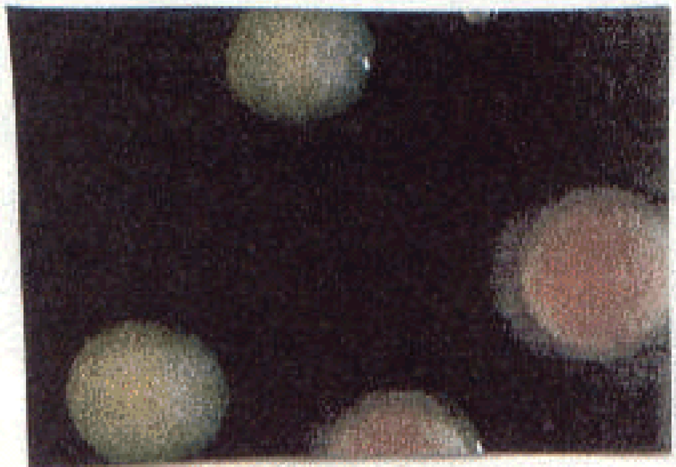
Salmonella possess both O (somatic) and H (flagellar) antigens like the E. coli. Many types can have two forms of flagella which may occur at different phases which resulted in their being identified as group and specific antigens and group and specific phases. Now they are simply described as phase one and two. The presence of certain O antigens help inhibit phagocytosis and interferes with the bactericidal effect of blood serum and these make strains which possess them more virulent. The LPS content also affects the ability of neutrophils to produce antibacterial activity which protects the organism.

The H antigens are heat labile and are broken down by ethanol or acid as well. They may occur as either phase and both may be produced by a single parent strain in different progeny during subculture.

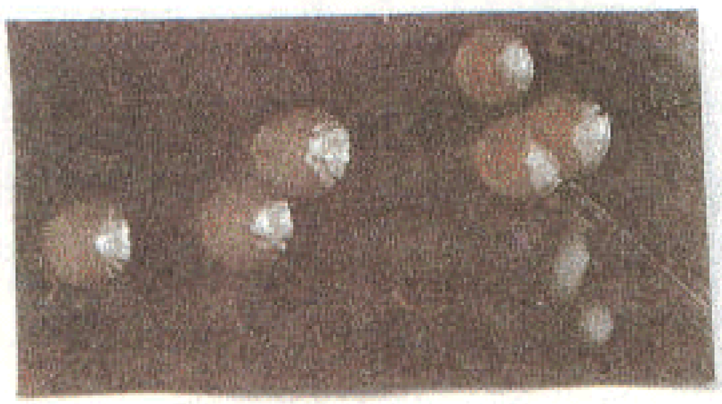
Some Salmonella, especially S. typhi also have the Vi antigens which is directly related to the virulence of the organism. It is a surface antigen that can mask the O antigen in a manner similar to the K antigens in E. coli. It is detected by boiling for 30-60 minutes which destroys them and unmasks the O antigens. The Vi antigens are often lost in continuous lab media subculture and is not recommended for bio ordnance.

All Salmonella form **endotoxins** (LPS) on their outer membrane (the O antigens). They also produce **enterotoxin** similar to that of E. coli LT and ST toxins and Cholera toxin during gastroenteritis which was finally isolated and described in 1975. It is also genetically and antigenically related to both of these toxins. It is a heat labile protein that induces fluid accumulation in the rabbit ileal loop and stimulates adenyl cyclase. There are many types of O antigens and any bacteria may contain more than one of them, and they tend to appear in recurring combinations. Most of the S. enteritidis produce both toxins.

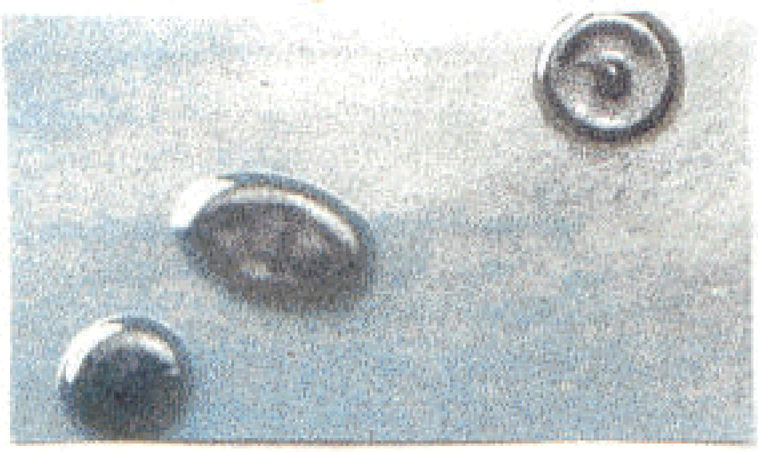
Creamy white Salmonella colonies with lactose fermenting pink E. coli.



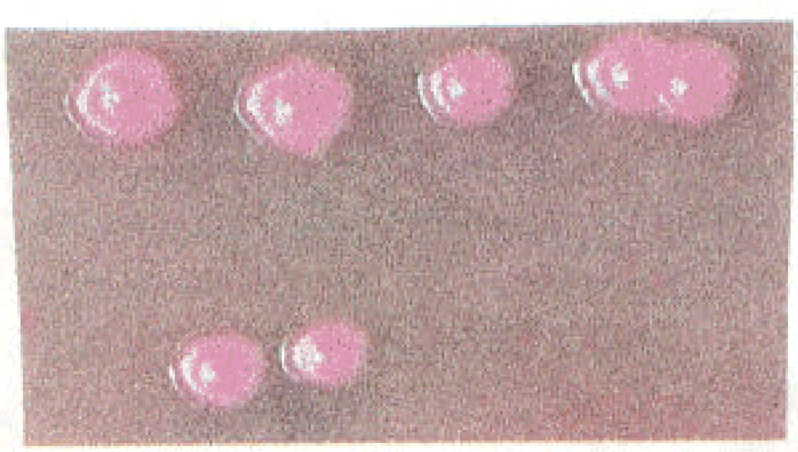
Salmonella with a beaten copper surface and slightly irregular edge.



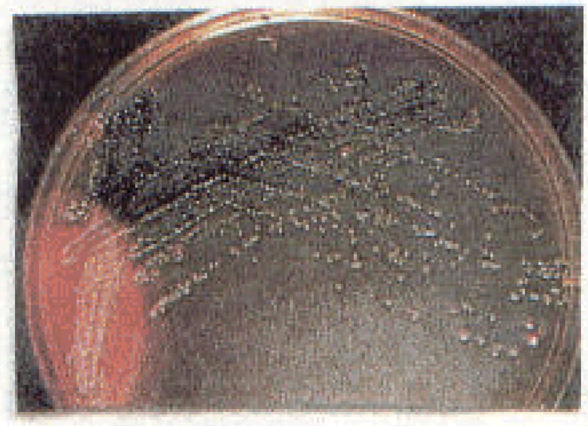
S. paratyphi growing as a mucoid colony which flattens over time. All three stages are shown.



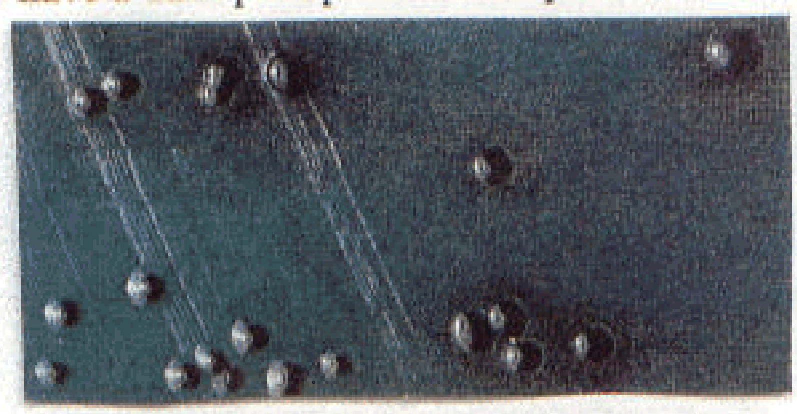
S. arizona on DCA agar, one of the few lactose fermenters among the Salmonella species.



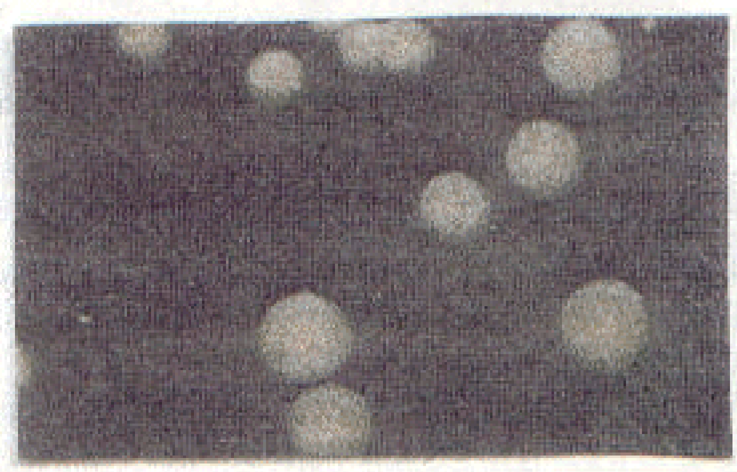
Black colonies are Salmonella with pink showing lactose fermenting E. coli.



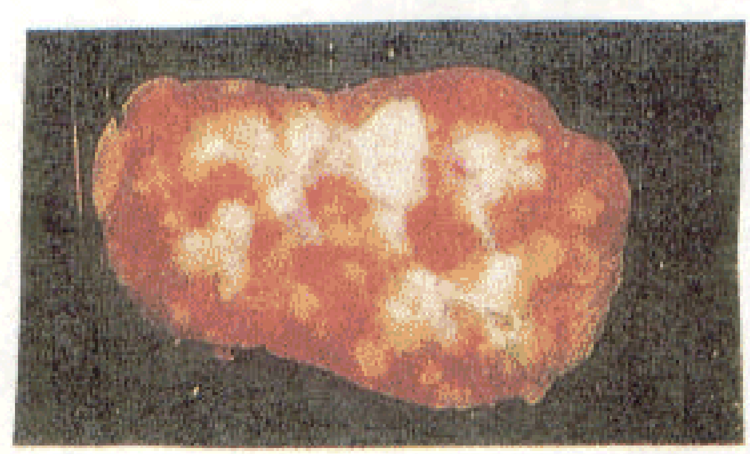
Colonies of S. typhi, some are conical and smooth (virulent). They have a central black core and clear peripheral zone. Others have a dark precipitate and tops are flatter.



S. typhi on DCA agar showing fine regular granularity under transmitted indirect light.



Spleen of a Salmonella infected Guinea Pig. Lesions are similar to those induced by tuberculosis.



When mice are injected with the neurotoxin, they are in a state of stupor with labored respiration, moderate diarrhea, and often with posterior limb and tail paralysis. They all died in a hypothermic state within hours to days depending on the dose. The LD50 for mice varied from .014 mg N to .04 mg N depending on the originating strain used. Intravenous injection is 4 times as effective as intraperitoneal in producing the effects.

Protection and Resistance

S. typhi are resistant to sulfonamides and penicillin's but are usually sensitive to chlortetracyclines. They are also resistant to many dyes which is why they are incorporated into selective media's (brilliant green, malachite green).

Vaccines have been developed for the specific antigens and seem to be effective only against low numbers of infective organisms. They are used commonly for westerners traveling to areas endemic with typhoid. Health officials try to detect, control, and treat the carriers, especially those in the food industries, in order to limit typhoid. The organism itself does not seem to stimulate effective immunity by itself and repeated attacks are known with the same strain in the same individual.

Vaccines are prepared for parental administration by killing the Salmonella with heat and preserving them with phenol or inactivating them with acetone. The suspension is then injected parenterally. The acetone inactivation worked the best in military field trials with protection ranging from 50-94%. The immunity can be overwhelmed by large numbers of infective organisms however. Attenuated live oral vaccine has shown some promise with protection against low numbers at 100% in children.

Treatment usually involves bedrest and convenient access to the bathroom. Light liquid diets and antibiotic therapy can deal with most cases not involved in actual weapons attacks.

Chlorination of water supplies and pasteurization of foods and other ingested liquids is mandatory to limit outbreaks. Heating to 140 F kills Salmonella while refrigeration at 40 F halts reproduction. S. typhi carriers usually have their gall bladders removed to eliminate the carrier state and this is effective in about 70% of the cases.

Incorporation into Weapons

S. typhi and other Salmonella are easy to mass produce and deliver in volume. They make ideal weapons when used after flooding occurs. This makes it indistinguishable from natural spread and conceals the use of the organism as a weapon.

Shigella

History and Recovery From Nature

Shigella primarily infect only humans which provide the principal reservoir although some other primates have reportedly been colonized. During the 1980's, the author in a few instances recovered Shigella organisms from swine herds in Nebraska. Shigella is the primary cause of dysentery and during the US civil war, more soldiers died from dysentery than from battle making it a potentially more effective battlefield weapon than guns. It also proved to be a serious problem during both world wars and in other operations like the US landings in Lebanon in the 1950's.

The first dysentery bacilli to be described was found in 1898 in Japan by the bacteriologist Shiga and was then referred to as the Shiga bacillus. It became later known as the group of organisms known as Shigella.

Genetically, the Shigella are closely related to E. coli, belonging to the tribe Eschericheae. There are four principal species, all of which can cause dysentery which will vary according to the species. These are S. dysenteriae, S. flexerni, S. boydii, and S. sonnei.

From 1964 to 1973, there were more than 105,000 cases identified in the United States. Of these, 73.7% were S. sonnei, 35.1% S. flexerni, .7% S. boydii, and .6% S. dysenteriae. The cases of S. sonnei has risen in recent years to more than 80% while almost all cases of S. dysenteriae originate from overseas. Tow thirds of all these cases occurred in children under the age of ten.

Humans are the main reservoir and source of infection and spread of the disease. It is found throughout the world and is common in third world countries. The carriers shed the organism in their feces where the cells are spread by flies, fingers, or food. Shigella has been isolated from toilet seats, contaminated water, and clothing of carriers. Most outbreaks occur in closed groups such as mental hospitals, indian reservations, day care centers POW camps, and in third world countries with contaminated water supplies. In 1967, there were more than 1,350 cases of Shigella per million cases compared to 5.9 cases per million in the general population.

It has been found that males are more frequently infected than females except in the 15-45 age group where intimacy of contact between mothers and daughters produces greater exposure to women than men. The incubation period is about 48 hours.

In the tropics, outbreaks are most common in the warmer months while in the mid and northern latitudes, outbreaks occur mostly in the late winter and early fall.

All Shigella species cause bacillary dysentery, a watery and painful diarrhea that is tinged with blood, mucus, and groups of polymorphonuclear leukocytes. The organism enters the small intestine, multiplies, and then moves on to the terminal ileum and colon where it invades the epithelial cells and multiplies. This causes inflammation, sloughing of cells, and superficial ulceration. The organism rarely penetrates through the intestinal wall to spread to other parts of the body. If septicemia does occur, many complications can set in with a mortality rate approaching 50%. Of all the Shigella, the mortality rate is highest with S. dysenteriae, reaching 25% in children, the old, and the malnourished who usually succumb to the effects of electrolyte imbalance and dehydration.

Many patients excrete Shigella in their feces for weeks and some are persistent carriers accounting for its continued occurrence and outbreaks. During the watery period, the Shigella are often recovered in immense numbers in nearly pure culture, crowding out virtually all other species. When complications set in the Shigella may also be responsible for other symptoms such as arthritis.

All Shigella do not have flagella, are non-motile and do not produce H2S. They do not produce gas during carbohydrate fermentation except for a few types of S. flexerni. These characteristics allows them to be differentiated from Salmonella, while they are distinguished from E. coli by the fact that they do not produce lysine decarboxylase, utilize acetate as a carbon source, or ferment lactose rapidly.

S sonnei will ferment lactose after long incubation periods which may make it similar to E. coli in culture media.

Some noninvasive mutant strains cannot produce disease. The S. dysenteriae type 1 and S. flexerni are known to produce enterotoxins in broth cultures. It is not yet known if an intracellular toxin is produced and excreted by Shigella.

Shigella are most easily obtained by taking samples of fecal material from infected individuals. This must be done quickly with culture media being inoculated immediately because the Shigella are sensitive to acids in the fecal material. If this is not possible, the sample should be placed in buffered glycerol media for transport.

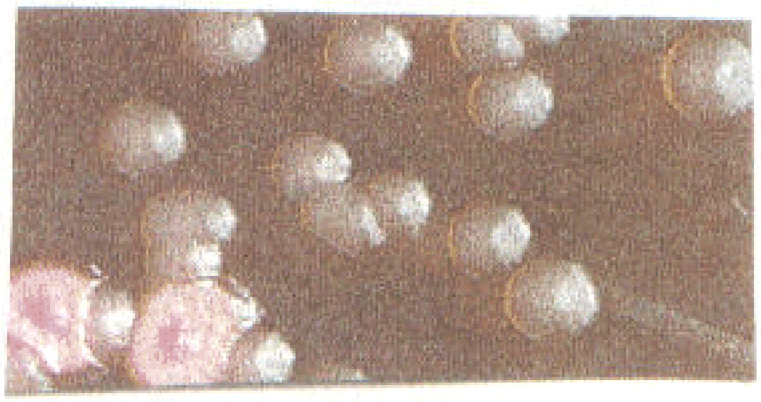
The individual types are separated as follows -

	Glucose	Lactose	Mannitol	Indole
S. dysenteriae	A-ng	-	-	V
S. flexerni	A-ng	-	A-ng	V
S. boydii	A-ng	-	A-ng	V
S. sonnei	A-ng	A-ng	A-ng	-

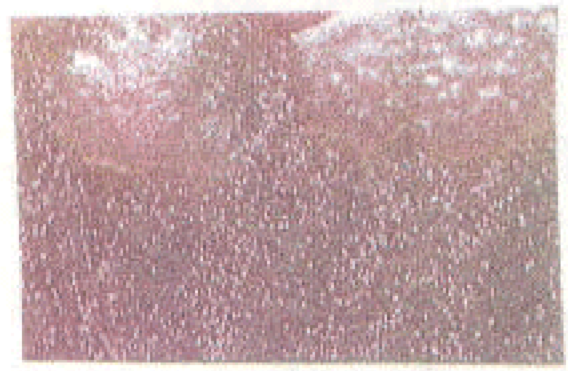
A-ng = Acid produced, but no gas.

The S. dysenteriae is set apart from other Shigella by its ability to ferment mannitol. The S. sonnei are distinguished by their fermentation of lactose which is slow and may be delayed for a week to ten days which will cause them to be confused with other species.

S. sonnei on DCA agar with smooth colonies (virulent) and the "coolie hat" shape. The pink colonies are coliforms that ferment lactose.



Rough, non virulent S. sonnei on MacConkey agar with large volumes of fecal streptococci colonies. The S. sonnei are rough, flat and effuse, spreading, irregular edge.



<u>Growth and Production</u>

Shigella tolerate low temperature environments when adequate moisture is present. Their terminal death point is 55 C for one hour. On blood agar their colonies resemble E. coli. They grow from 10-40 C except for S sonnei which will grow at 45 C. The pH range is 6-8 and they are partially inhibited by bismuth sulfite and brilliant green. Shigella do not grow on citrate agar, are urea negative, methyl red positive, and Voges-Proskauer negative.

They are gram negative, non spore-forming rods measuring .5-.7 *u*m in width by 2-3 *u*m in length that are closely related to the other enteric bacteria. They ferment carbohydrates with production of acid but no gas in a manner similar to the typhoid bacillus. They are aerobic and facultatively anaerobic and grow optimally at 37 C. They grow on all ordinary nutrient media containing peptone and beef extract. They ferment glucose without gas producing lactic acid, and some formic and acetic acids as well as ethyl alcohol. They are resistant to dyes that are added to media to isolate them from other organisms.

The smooth colonies are generally the most virulent due to the presence of the full compliment of antigens. These are most often recovered when grown on SS, DCA, or Bismuth sulfite mediums which are used for selective recovery of Shigella.

Shigella may be found viable in tap water for up to 6 months and milk supports their growth. They have been recovered from soiled clothing weeks after contamination. They are found in the pus and mucus in large numbers in stool samples but are found rarely in the liquid portion.

Toxicity and Harm

Shigella invade the body by first penetrating the epithelial cells of the mucosal surface of the terminal ileum and colon. After the bacteria multiply in the epithelial lining, a local inflammation occurs followed by cell death and sloughing of the lining.

All Shigella possess O antigens and some possess K antigens. Those with K antigens produce smooth colonies when grown on agar. When the K antigen is present, it interferes with the serologic typing of the O antigen. This interference is usually eliminated simply by boiling the culture in water suspension for 30 minutes.

The Shigella are divided into 4 major O antigen groups which each represent the four types, dysenteriae, flexerni, boydii, and sonnei. These are further subdivided according to the exact type of O antigen they possess. There are about 40 serological types of Shigella known.

The O antigens are antigenically similar or identical to those of other enteric bacteria so they must first be identified correctly as being Shigella before serological testing can be meaningful.

The Shigella produce an endotoxin (LPS) which is responsible for the O antigenicity of the bacillus. This makes the cell substance (cell wall) alone a toxic material. These toxins are very similar to the LPS toxins of the other enterobacteriacaea.

The Shigella are unique in that they also produce an exotoxin that is neurotoxic. It was first observed in 1903 to cause paralysis when injected into rabbits. Enterotoxins produced by the Shigella appear to be similar to the neurotoxins described for E. coli LT and the Vibrio cholera enterotoxin. They are heat-labile and cause fluid accumulation in the ileal loops of rabbits. In classic bacillary dysentery, there is little evidence of the activity of this toxin in the large bowel. It seems to be mainly active in the small intestine. It is believed that the watery diarrhea that occurs at the onset with Shigella infections is due to the effects of this toxin and this changes to the classic dysentery when the infection moves into the colon.

Various crude enterotoxin fractions have been shown to be toxic for HeLa cells and cytotoxins have also been isolated and identified. People infected with Shigella produce antibodies which neutralize the effects of the cytotoxins. Their actual role in the disease process is not actually known.

In 1905, Krauss and Dorr found that Shigella produced two toxins. The first was a soluble toxin that was present in 8-10 days in filtrates of cultures that was fatal to rabbits but not guinea pigs. It also caused the production of specific neutralizing antibody. The second was an insoluble toxin present in the cell bodies (the LPS) that was fatal to both rabbits and guinea pigs.

The neurotoxin exotoxin affects the nervous system of experimental animals to induce paralysis and subsequent death. It is believed to be partly responsible for the more serious cases of dysentery. It is also very toxic, about 1,000,000 times as deadly on a same weight basis as strychnine (page 2-81). It first produces flaccid paralysis in the front legs and then spreads to the hind limbs with the animals sometimes living as long as 10 days after the first symptoms. On autopsy it was found that the spinal cord grey matter had softened and their were small hemorrhages in the brain.

Their are no symptoms in the rabbits for the first two days following an injection of 1-2 LD50's. The paralysis then sets in and by day 5 complete paralysis sets in with complete loss of muscle tone. The animal is usually unable to hold its head up. If the animal survives, even from complete prostration, it can sometimes achieve a rapid and complete recovery. Their are a number of changes to the nervous system, most evident in the spinal cord and usually evidence of widespread destruction is apparent. Blood vessels are also affected making it a potent vascular toxin as well.

The toxin is composed of several parts including one heavy (A) chain and 6-7 light (B) chains. The A chain is the active portion inhibiting protein synthesis in susceptible cells while the B chains are believed to act as binding units by reacting with receptors on cell surfaces.

Intravenous injection of Shigella cultures in lab animals produces a hemorrhagic enteritis and if the animal survives, muscular paralysis is observed. Oral administration shows no effects on test animals. Primates are the only animals affected by oral administration of Shigella species, however it has been discovered that animals may be pretreated to increase susceptibility and then be successfully infected with the bacilli. This pretreatment usually involves starving with opium as a part of the diet.

The animal test for invasiveness is accomplished by instillation of the organism into the eye of the guinea pig. If the bacilli is capable of invading human tissue, it usually causes keratoconjunctivitis (called the Sereny test).

The two toxins are easily separated by trichloroacetic acid. The neurotoxin is protein in nature and precipitates in the acid while the enterotoxin is soluble in it. The neurotoxin is produced by all Shigella colony forms while the enterotoxin is produced only by smooth colonies.

Culture filtrates of Shigella are not very toxic even though the toxin is deadly in tiny amounts. This is due to the very low amounts of toxin actually produced (about 1 mg per gram of dry weight of organism or .1%). Only a fraction of this toxin passes through the cell wall into the medium further diluting it. This reduces the culture filtrate toxicity to about 250 MLD mouse per ml of filtrate. Upon purification, the toxin has been reported to kill when injected intraperitoneally at an LD 50 of .027 *u*m per kg of mouse and about .00087 *u*m/kg of rabbit. This toxicity is about equivalent to that of botulinum or tetanus in guinea pigs and 350 times that of the diphtheria toxin.

Now free of nucleic acids, the supernate is freeze dried and the powder dissolved in 1% salt to a concentration of 5 gm. per 100 ml. The toxin is insoluble in the absence of salt so the solution is dialyzed at 4 C against several changes of distilled water. Most of the toxin precipitated out over 10-20 days. It was then dissolved in .1 of the original volume of the 1% salt solution leaving a significant insoluble residue which is discarded.

A copious precipitate forms after about 30 minutes and is dialyzed overnight. This precipitate is collected, redissolved, and redialyzed. After two more dialyses, the preparation still contains some impurities. The remaining impurities were removed by adsorption onto calcium phosphate, precipitated as in the earlier stage. The supernate was dialyzed and freeze dried to yield a highly concentrated toxin.

The exotoxin of S. dysenteriae is a protein with a N content of 15.7%, is insoluble in distilled water, and is soluble in aqueous salt solution, with a high temperature coefficient. Cooling a 1% solution of toxin in 1 % NaCl to -5 C, a precipitate forms that redissolves on cooling.

The toxin is best produced in alkaline broth's and can also be released into the medium by the use of bacterial phage which kills the cells and causes lysis.

Protective Measures

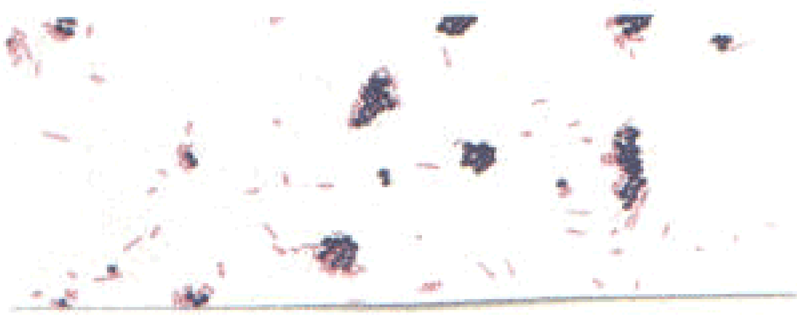
Shigella are more sensitive to antibiotics, acids, high concentration of biles, and disinfectants than most other enterobacteriacaea. The use of antibiotics reduces the severity and mortality of the disease, especially in children. Tetracycline, ampicillin, streptomycin, chloramphenicol and trimethoprim-sulfamethoxazole have good track records but some resistant strains have been isolated in recent years.

Insuring that plenty of fluids are available and that electrolytes are kept in balance to avoid the most deadly effects of the infection. Adequate sanitation, pasteurization, early detection and treatment are the best public health methods of dealing with outbreaks. Use of chlorinated water is very important since chlorine kills Shigella very quickly. Boiling contaminated clothing or soaking in chlorine may be necessary when working in contaminated areas.

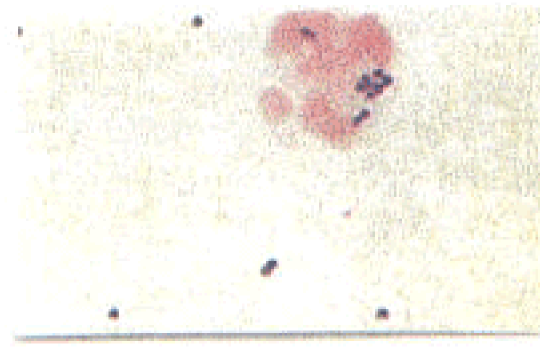
The use of foot operated or infra red detection operated toilets and sinks limits disease spread. This leaves toilet seats as the primary contact spread in restrooms. In field situations, the use of bored hole latrines with chemicals added to soak the feces reduce the survivability of o the organisms and prevent their spread by flies and other insects.

Vaccines that raise humoral antibodies are not effective and it is presumed that this is due to the localized nature of the infection. The body does produce antibodies to Shigella and its toxins to low titer in a few days after infection and this accounts for the milder symptoms seen by much of the population.

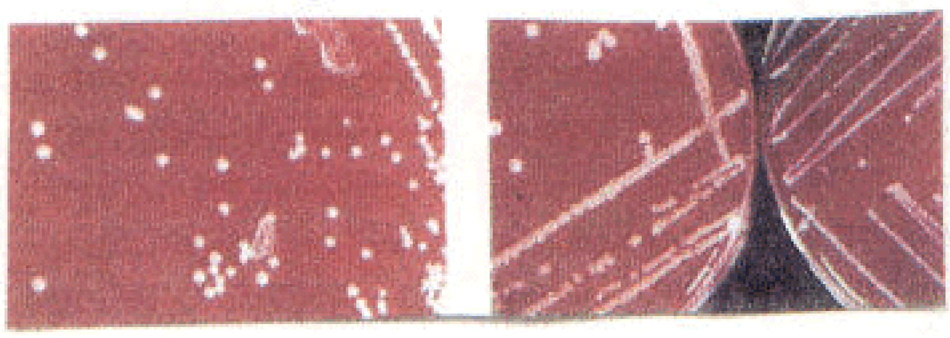
A gram stain of a mixed culture. E. coli stains red while S. aureus stains as purple spheres in grape-like clusters.



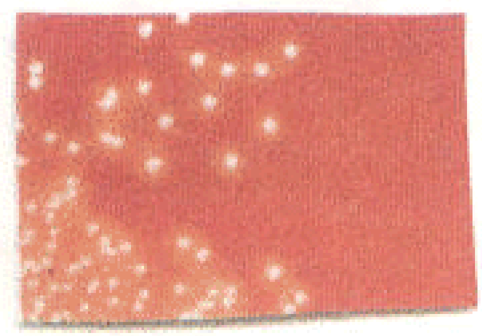
Pus from an abscess in which a cluster of S aureus is seen. Pairs and singles are also seen.



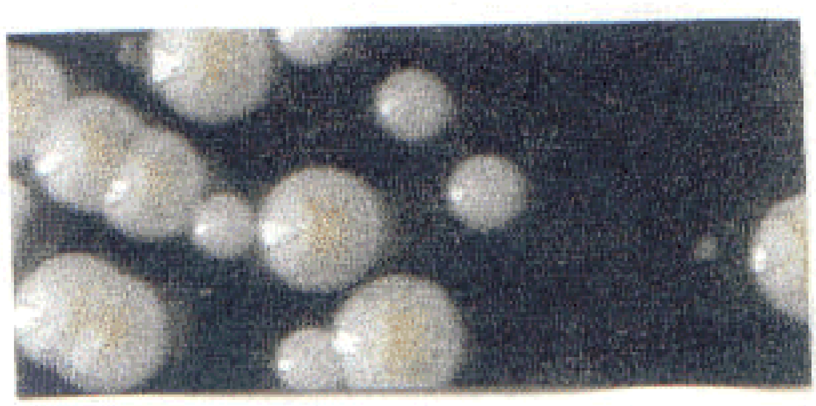
Blood agar with staph colonies on the left and plates side by side showing S aureus-left and S epidermi-right.



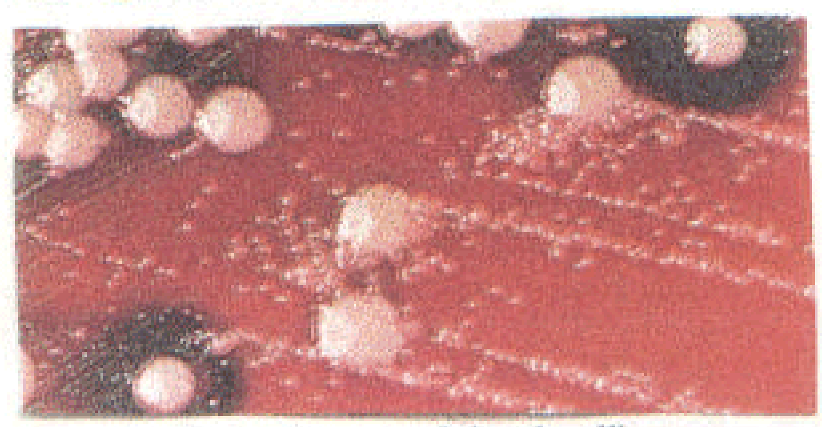
Beta hemolysis surrounding colonies of S aureus.



S aureus with the deep gold pigment in the center. The smaller colonies without pigment are S. albus.

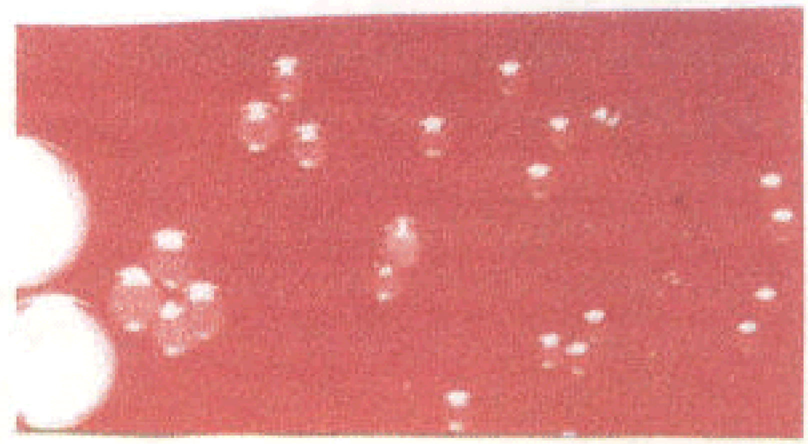


S. aureus and the smaller diptheroids cultured from a nasal swab. Notice two types of S. aureus. A strain that produces the dark zone of hemolysis also inhibits the diptheroids while the other strain doesn't.

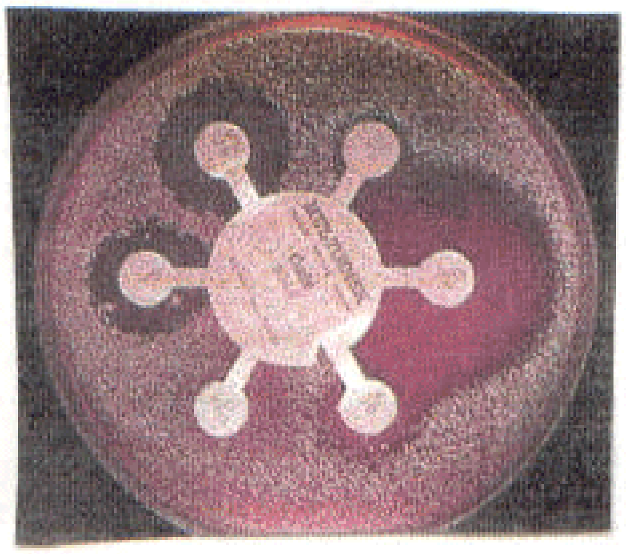


When the author grew livestock disease organisms in the 80's, one of the deadliest in swine operations was haemophilus influenza which could also infect and kill people. It required a special "V" factor which was not contained in blood in order to grow. S. aureus produces this V factor which enables it to grow on blood. Notice that it only grows near the S. aureus colonies. Combination of this type make excellent weapons.

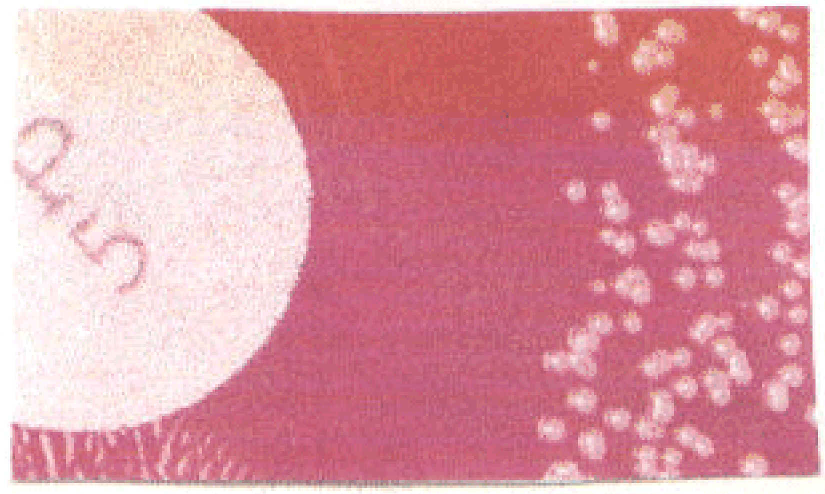




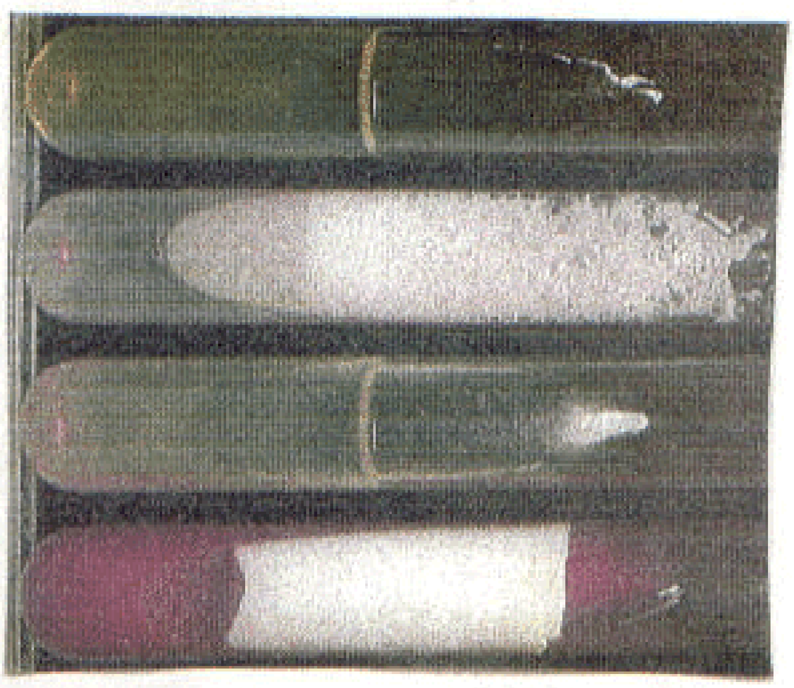
A hospital S. aureus strain showing resistance to many antibiotics that are on the different discs.



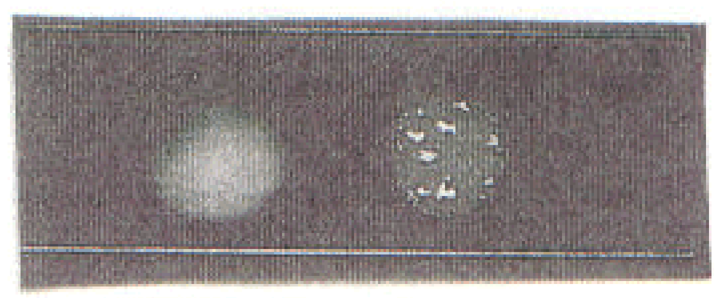
A strain of penicillin resistant S. aureus growing near the penicillin disc. A spreading growth of proteus is near the disc.



Catalase is an enzyme that converts hydrogen peroxide (3% added) to water and free oxygen. Bubbles of oxygen produced are seen -
Top- Control tube No growth
Second- Positive bubbles from S aureus
Third- Negative from Streptococcus
Bottom- False positive from blood



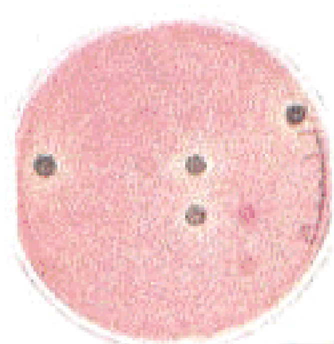
Slide coagulase test in which S. albus on left and S. aureus on right are mixed in water to form emulsions. A loopful of human plasma is added to each ans stirred. In five seconds, the cells of the S. aureus clumped the blood (coagulase positive)



Growth of S aureus on mannitol salt agar in which the colonies turn yellow.



S. aureus producing black colonies due to tellurite salts added to the medium.



Staphylococci

History and Recovery from Nature

The staphylococci were among the first bacteria to be recognized as being capable of causing disease and were first characterized in the 1880's. They are the bacteria most often isolated from human specimens because they normally live as commensals on our skin, groin area, perineum, and in the upper respiratory tract. Infants are colonized soon after birth by contact with attendants and family as most staph is transmitted by direct contact with the hands.

The Staphylococci are also often found specifically in the nasopharynx of healthy individuals, especially hospital workers. Most of the strains are opportunists and only invade tissues when we are injured. Colonization with one strain of staph often interferes with colonization by a second strain which aids in resistance to infection by new, virulent staph. This interference appears to be due to competition for the receptor sites necessary for the bacteria to adhere to. Whoever gets their first usually wins and the winning strain usually has to be removed by antibiotics or other effects before another strain can colonize in its place.

The ability of these organisms to invade depends somewhat on their ability to produced the enzyme coagulase and it is this ability to produce it that differentiates these species. The staphylococci capable of producing this and other toxins for use in weapons are S. aureus, S epidermis, and the micrococcus species of which most are not harmful. The S. aureus name comes from the golden yellow pigment produced by the pathogenic colonies. Infections arise most often when trauma, skin lesions and burns are involved allowing the organism to enter deep tissues where it can grow. Skin irritants like oil and grease also can cause superficial infections.

When the staph first invade and begin to grow inside human skin tissues or mucus membranes, our phagocytes migrate into the area to stop it. The accumulation of phagocytes starts a war and this interaction determines how far the infection will spread. If the staph produces effective anti phagocytic toxins it may likely win. If they do not, they are quickly engulfed by the phagocytes and are killed very rapidly. Some strains stay alive inside the phagocytic cells. The strains with these properties tend to produce persistent infections in their hosts and make more virulent weapons.

A central necrotic core is formed with dead leukocytes and bacteria which are separated from the surrounding tissues by a fibroblastic wall formed by the coagulase enzyme. This inflammation will either remain local or spread into the circulatory system and reach most other organs and tissues. The initial infection yields some redness, swelling, and some pain in the first few hours. In 3-5 days, an increase in necrotic material and liquefaction in the lesion causes a rise in pain, tension, and tenderness. The apex of the swelling, the skin glistens, becomes thinned and soon drains spontaneously. The abscess is ruptured and a creamy yellow pus is drained with the pain stopping almost immediately.

The organisms are spread by fomites, contaminated gauze, handkerchiefs, and many other contact materials and clothing that is handled during illness, especially in hospitals. In fact, hospital workers with mild staphylococcal lesions have been identified as the source of major epidemics. The organism is recovered from these items as well as pus, nasal swabs, wound, rectal, and ear swabs, blood, sputum, and even spinal fluid. Since the organisms are so resistant, no special media and conditions are required to preserve them. They are responsible for about 80% of the skin infections encountered in medicine. Most infections occur from damage to the tissues through other infection, surgery, wounds, skin ulcers, and especially burns. [Vacuum cleaners in hospitals are excellent sources of virulent strains of many organisms]

Under the microscope, the staphylococci are gram positive cocci that divide in two planes making them appear in grape like clusters (staphylo means "bunch of grapes") when grown in solid cultures and this is how the name classification came about. Their average diameter is about 1um and varies with the age and type of culture media used. Older cultures have more single and enlarged forms and may stain gram negative. In broth cultures they occur in singles, pairs, and odd short chains. Longer chains are rarely seen.

Staphylococcus are aerobic and facultatively anaerobic, growing well on simple media at 7-42 C and a pH of 4.2 to 9.3. Optimal growth temperature is 36 C and best pH is 7-7.5. Some strains show improved growth with increased CO2 in the medium or atmosphere.

Their colonies on agar are large measuring 2-3 mm in 24 hours at 37 C, entire, shiny, circular, convex, domed shaped, opaque, butyrous, with a creamy consistency when poked with a wire, and frequently pigmented ranging from creamy white to deep golden. They are strongly beta-hemolytic on blood agar and are strongly catalase positive. This is a test that is done by taking a colony and adding a drop of hydrogen peroxide (3%) to it on a slide. Organisms that can produce catalase (by decomposing the peroxide) release gas bubbles when the peroxide is mixed into the colony. Similar looking colonies of streptococci are catalase negative. They do not release gas. Nitrates and methylene blue are reduced and urea is hydrolyzed by staph strains.

These organisms produce a variety of different toxins. An enterotoxin is responsible for staph food poisoning and ingestion of small amounts of these produce vomiting and diarrhea in 4-6 hours. An exotoxin causes necrosis of the skin and lyses red blood cells while producing boils and other local abscesses. From these local inflammations, the staph may spread by way of the lymphatic and circulatory systems. If this occurs it often leads to much more serious disease like pneumonia, meningitis, endocarditis, etc. Staph pneumonia is rare except during flu epidemics where an established infection becomes very deadly due to the ability of the staph to produce abscesses and destroy lung parenchyma.

S. aureus is the most potent toxin producing member of this group and it is easily identified by growing it on blood media with mannitol and a tellurium salt. The mannitol is turned yellow in a halo around the colonies and like the C. diphtheria, the S. aureus reduces the tellurite to free tellurium which turns the colony black.

The S. aureus species is the only one of this genus that produces different (free or bound) coagulase enzymes that causes blood to clot. This is a significant advantage in that clotting blood cuts off circulation allowing growth of first aerobic, and then anaerobic organisms. It also provides a solid blood medium on which to grow that cannot protect itself from breakdown of its constituents into bacteria food.

A slide test is performed by mixing a drop of S. aureus colony into a drop of water on a microscope slide and mixing in a loopful of rabbit blood plasma (human blood may also be used). The appearance of white clumps within five seconds indicates a positive test. Only "bound" coagulase or clumping factor is detected by this method. The tube test is performed by mixing coagulase rabbit plasma (1 part) with four parts water and then inoculating it with the colony. Positive strains produce a positive test by clotting the blood in 1-4 hours. This should be read at 4-6 hours and 16-18 hours however because some strains produce coagulase very slowly while others produce a fibrinolysin that redissolves the clot by the next day which also helps in identifying the best toxin producing strains.

These tests are important because the combination of beta hemolysis on blood agar combined with positive coagulase means that these S. aureus are among the most virulent strains. A few strains produce a capsule or slime layer that also enhances its virulence.

A selective medium used to recover and easily identify S. aureus is to make a blood or other medium and mix in 7.5%-10% sodium chloride, .025% phenol red for color change, and 1% mannitol. The high salt content inhibits most other organisms while the Staph family grows well on it. The S aureus strains ferments mannitol unlike S. epidermidis and the micrococci.

Most of the Staphylococcus on the skin are coagulase negative S. epidermis. The coagulase positive S. aureus is usually found colonizing the nasopharynx and those that are recovered from the skin are usually contaminants from nasal secretions. Hospital air and dust samples contain large amounts of S. aureus.

Staphylococci are considered to be opportunistic pathogens and cause infection when injuries occur or when the strain possesses the combination of virulence factors that allow invasion into susceptible host tissue. The principle lesion caused by staph is called an abscess, which is a localized focus of purulent infection that is partially or completely walled off from the surrounding tissues. Many of the infections start as simple boils or acne that may spread from the abscess and disseminate widely, especially in children.

The most common form of infection is cutaneous where the organism invades the hair follicles or sweat ducts. The bacteria can spread to the bloodstream causing infection in multiple tissues and organs. If they penetrate the mucous membranes, they may cause pneumonia. The pneumonia is rare, usually following a bout with the flu as a secondary infection. Its use a an added infective weapon is recommended because when its invasion of the mucous membranes is successful, the mortality rate is high.

Growth and Production

Staphylococcus are among the most resistant of the non sporing bacteria to the envoirenment. Stored in agar they may remain viable for years and are resistant to heat, requiring more than 80 C for 30 minutes to one hour while in suspension to destroy them. In the absence of serum, pus or albumin which strongly preserves them, they are killed by exposure to 2% phenol in 15 minutes, 3% hydrogen peroxide in 15 minutes, and by 70% ethanol in one hour. They survive for weeks or months in dried fluids and tissues. Most solutions that contain free chlorine like sodium hypochlorite will quickly kill the organisms. Most organic dyes are also bactericidal to the staph organisms.

The cells grow anaerobically at 30-37 C as well as under the aerobic conditions described earlier. Their colonies grow from .5-1.5 um in diameter, are non motile, and do not form spores. The colonies most often form nearly perfect spheres measuring 1 um in diameter. The cell grouping that gives it the grape-like clusters comes from the geometry of cell division in which daughter cells are translocated by a separation enzyme to produce the typical irregular clusters.

The usual meat extract and/or peptone media may be used in place of blood which is ideal for its growth. Synthetic mediums require will grow staph with the basic amino acids and vitamins but anaerobic growth requires added uracil and a fermentable carbon source like pyruvate. Carbohydrates are used in aerobic cultures with acid being produced. Glucose is fermented anaerobically with lactic acid as the end product. Catalase is produced when grown aerobically. S aureus ferments mannitol and S. epidermis does not which aids in identification.

S. aureus grows in salt broth 10%, or 7% salt milk agar used to select out most other organisms. A uniform turbidity forms in broth cultures. Confluent growth on agar appears like "oil paint". Blood agar produces larger colonies than salt milk or nutrient agars. With neutral red indicator in the medium, the colonies will turn pink from acid production. Gelatin is liquefied quickly while coagulated serum liquefies slowly. On milk agar, the pigment forms more rapidly and is more intense. Areas of clearing around the colonies is due to digestion of heat coagulated casein in the milk by the proteases.

Adding glycerol monoacetate 1% to nutrient agar allows enhanced pigmentation that makes it possible to group various staph strains. Yellow colonies are produced by pathogenic antibiotic resistant strains while orange and buff colonies are intermediate in virulence. Growth on milk or egg yolk may produce zones of opacity or clearing due to the lipase activity of the strain. With both glucose and egg yolk in broth a dense opacity is produced due to lipolytic activity and coagulase negative and most animal strains do not produce this reaction. By adding plasma or fibrinogen to the media, a coagulase enzyme reaction may be observed as a white halo or ring of opacity. By adding a trypsin inhibitor (from soybeans) the fibrinolysin can be prevented from digesting this opacity and producing a zone of clearing.

Although they do not form spores, a few strains produce capsules. They grow abundantly on most agar mediums with a pigment ranging from golden yellow to lemon yellow, to a creamy color. Pigmentation caused by cartenoid pigments occurs most often in the primary isolated colonies and is best observed after growth on agar for 24 hours at 37 C followed by incubation at room temperatures for 24-48 hours longer. No pigment is produced under anaerobic culture. On blood agar, most S aureus and S haemolyticus colonies are surrounded by a zone of B-hemolysis. A-hemolysis is not seen in staph species.

Most strains are very resistant to drying out and this allows them to be grown at the unusually high temperature of 45 C. Most strains grow in 10% salt and many will grow in up to 15% salt concentrations. This is why salt preservation in foods sometimes fails as these strains of staph can grow and form enterotoxin in conditions that stop most other organisms. Freeze drying in broth or culture allows for survival of strains for years. Strains also survive in fabrics (especially in hospitals) but are slowly killed in sunlight.

Toxicity and Harm

Coagulase is a protein enzyme that has a prothrombin like activity that makes it able to convert fibrinogen into fibrin which causes the blood to clot. In Staph infections this allows the organism to produce a fibrin barrier at the site of the growth that protects it and provides a ready made solid blood media to grow on. It also serves to localize the infection into abscesses (like carbuncles and furncles) which protects the body against further extensive invasion.

S. aureus is the only species that produces the coagulase enzyme as well as its hemolytic toxin. These two in combination usually determines how virulent the strain is. Encapsulated strains are poorly attacked by phagocytes making these strains more virulent and better weapons as well.

The following toxins produced by the Staph species are-

Leucocidin which is cytotoxic for all rabbit and human leucocytes and is dermonecrotic in rabbits. It is antigenically distinct and is converted to toxoid by formaldehyde. It is composed of two parts called S (slow) and F (fast) which by themselves are not toxic. When white blood cells are treated with leucocidin, their cell walls become more permeable leading to a loss of motility, swelling and losing its shape, granules forming around the cell periphery, and final complete disruption of activity. Human antibodies are produced in staph infections with this toxin and these aid in resistance to the infection. It also attacks macrophages but no other cell types.

The ability of this substance to destroy the leucocytes reduces the phagocytosis that the body uses to protect itself. This toxin would find use in inhalation weapons carrying organisms that are susceptible to attack by white blood cells because it would protect the organism from such attacks.

The crude toxin is isolated from culture filtrates by precipitation with ammonium sulfate followed by complete purification on calcium phosphate, carboxymethyl cellulose, Dowex, and Amberlite CG-50. It can also be directly adsorbed onto hydroxylapatite.

Most of the protein components precipitate with the toxin when ammonium sulfate is used so the concentration from crude material is very modest.

Hemolysins are produced by all the coagulase forming staphylococci which is seen as a clear zone of B-hemolysis on blood agar. These are soluble toxins that are excreted by the colonies and are present in cell filtrates. These consist of several distinct proteins called "staphylolysins".

> **Alpha hemolysin**- attacks a variety of cells including platelets, fibroblasts and leucocytes. It is produced by almost all strains of S. aureus. It is dermonecrotic, neurotoxic, and lethal in quantity. It is either proteolytic or acts on the surface of the cell membranes. It causes cell membrane damage that affects the central nervous and cardiovascular systems of animals. Human macrophages and platelets are damaged but monocytes are resistant. It injures the circulatory system, muscle tissues, and the renal cortex. No other bacterial toxin is so wide ranging and versatile in its overall effects. Its protease activity requires activation which occurs on contact with target cell membranes.

Upon injection in rats and other test animals, the subject makes uncoordinated movements, falls to one side and goes into convulsions with the extremities extended. They die within hours or days from respiratory distress.

Partial purification has been achieved using various amounts of trichloracetic acid, acetic acid, methanol 15 %, ethanol, zinc acetate and phosphoric acid precipitation at 4 to -20 C. To obtain complete purification, continuous flow paper electrophoresis and chromatography have been used. Total purification is impractical because the purified toxins are unstable and deteriorate completely in about three days even when frozen. Crude toxin is much more stable but is inactivated by heating at 60 C for 30 minutes and can be partially restored by further heating to 100 C for five minutes and/or treatment with urea (8 moles).

The first stage of producing crude toxin is by salting out with ammonium sulfate or methanol at pH of 4 at temperatures below -5 C. Further purification and concentration is achieved with fractionation using ammonium sulfate at pH 8.6. After the first stage, organic solvents don't appear to be of much help.

All hemolysin production is considerably increased by the addition of 20% CO2 to 80% air mixtures. Mixing CO2 into broth and agar also significantly increases yields. Using agar, cellophane, keiselguhr (diatamaceous earth), kaolin clay, filter paper and other absorbent materials allows the staph to excrete the toxin into the absorbent material upon which the toxin prefers to concentrate and accumulate. Horizontal shaking in broth cultures yields good toxin production while end over end shaking or vigorous shaking produces almost no toxin. A casein hydrolyzate medium with 30% CO2 produces good yields and has been used with yeast diffusate in broth cultures. Store bought yeast culture that is heated killed in the oven until a burnt smell permeates the air has also been used by the author with good effect. The yeast also eliminates the need for the additional CO2. All these methods are suitable for large scale toxin production.

[Adding the yeast is an excellent way to improve S. aureus growth and toxin production in all mediums]

Most toxin production and accumulation occurs at 6-10 hours with a leveling off during the remainder of the growth phase.

In attacking erythrocytes, the toxin first acts proteolytically, and the toxin is absorbed by the cells where it acts on and changes the cell wall membrane as it passes through. This causes the cells to leak potassium ions. Soon, hemoglobin begins to leak as well. Then unwanted ions begin to leak into the cell leading to its death. This is believed due to phospholipase activity in the toxin. When this toxin is injected into the circulatory system, there is a rapid drop in platelet count as a result. This is due to the platelets immediately aggregating with key components such as granules and vesicles dissolving quickly. When large amounts are injected, peripheral vasodilation occurs followed by recovery and then marked peripheral vasoconstriction which results in a drop of blood pressure, hypotension, and eventual death.

The dermonecrotic effect is due to the direct cytotoxic effects on the skin cells and vasospasms of local arteries and veins causing local ischaemia. This loss of local circulation aids in establishing organisms in the infection site and is probably effective in aiding anaerobic organism attack when included in those weapons.

Beta hemolysin- is produced by almost all S aureus. It is toxic to leucocytes, macrophages, and fibroblasts and is weakly lethal for rabbits. It acts enzymatically by hydrolyzing sphingomyelin and lysophosphatides. It requires magnesium ions and produces hot-cold lysis which occurs from incubation at 37 C to a drop to 4 C to as high as room temperature for several hours. The sphingomyelin degradation is the membrane lesion that leads to the hemolysis when the cells are chilled. Animal erythrocytes are sensitive to this toxin based on the sphingomyelin content of their cells.

Beta hemolysin was first purified by dialysis followed by absorption on hydroxylapatite. Another method involved a double precipitation with 65% ammonium sulfate solution followed by Sephadex G-100 column fractionation and final purification with DEAE cellulose fractionation. The crude toxins were dermonecrotic while the purified toxins were not and these could not be activated with the addition of magnesium ions which means that using the toxin in crude form on a carrier with some magnesium is best.

Crude toxin can be upgraded to modest concentrations by precipitating the crude filtrate using acetate, methanol, or ethanol at a pH of 9.0

This toxin can be inactivated by heating at 60 C for one hour while its ability to produce hemolysis in erythrocytes is intensified by the addition of magnesium and/or cobalt cations. Its activity resembles that of phospholipases. IV injection in rabbits cause rapid increase in heart rate to 200 beats per minute followed by slowing and irregular heartbeat which ceases before death.

The toxin first came to researchers attention when they noticed incomplete or absent hemolysis at 37 C but by incubating further at 4 C a darkened zone around the colonies converts to complete hemolysis. Concentric ring effects were noticed as the red cells in blood agars would lyse in addition to the effects of other toxins took place.

Alpha and Beta toxins are produced in volume by growing the culture in fluid or semi solid media with added CO2 (25%) in air or the medium. Toxin production is increased from 20 to 130 times over media and atmospheres without it. Beta toxin production is promoted by the addition of magnesium or manganese ions in the medium (anions are not mentioned in the literature). A pH of 6-7 is best with toxin production nearly non existent outside these ranges. Adding a buffer helps as the pH rises during production.

Adding nicotinamide and thiamin to the growth medium has been found to increase both growth and Beta toxin production. Nickel and Iron as well as the other mentioned metal ions enhance the activity of the hemolysin

Gamma hemolysin- is composed of two components that are cytotoxic to human leucocytes and lymphoblasts. It is lethal for guinea pigs and its mode of action is not published but requires sodium ions. It causes release of lysosomal enzymes which contributes to the infection. It is hemolytic on blood but inhibited by agar and membrane phospholipids.

Delta hemolysin- attacks the erythrocytes of primates, sheep, and rabbits. It is lytic for bacterial protoplasts and spheroplasts, lysomes and mitochondria. It inhibits water absorption in the ileum of rabbits and guinea pigs. It has a detergent like action on cell wall membranes and has been implicated in intestinal diseases. It is thermostable and its activity is inhibited by phospholipids and dilute normal sera. It also damages macrophages, lymphocytes, neutrophils, and platelets.

Delta toxin is produced best by growing the staph on the upper surface of cellophane sheets lying in contact with the medium. The toxin is retrieved after 18 hours incubation by washing the growth off the cellophane with saline and filtering with the fluid containing the hemolysin. When grown in broth, the media must be aerated to produce any yields of delta toxin. The toxin is produced in largest quantities when heart infusion is added to the media.

The purified toxin has been produced using chromatographic methods on calcium phosphate and TEAE cellulose columns. The protein is not thermostable and is destroyed by trypsin.

Extraction of the solids in the media containing the diffused toxin is accomplished by soaking in ethanol overnight at -4 C. After filtering, the ethanol is evaporated and then diethyl ether is used to extract the solids following the same procedure. This provides a significant two step recovery and concentration of the toxin. The delta toxin has also been absorbed onto alumina at pH of 8.0.

Enterotoxins produced by S. aureus are recovered in culture filtrates and cause illness on ingestion. These toxins are relatively heat stable proteins formed by the coagulase positive strains. There are six immunological types designated A, B, C, D, E, and F. The enterotoxins primarily affect primates causing projectile vomiting and diarrhea in a few hours. The symptoms usually subside in a few hours with few lasting affects. The toxic dose for humans appears to be 1-4 *u*m.

The toxins are purified to a crude form by ethanol precipitation and completely purified by gel filtration, starch electrophoresis, and chromatography. .05 mcg produces vomiting and diarrhea while 5 mcg produces emesis in monkeys. The vomiting can be caused by IV injection as well as ingestion.

The most interesting aspect of this toxin is its possible use as a non lethal vomiting agent. An accelerated version of this was depicted in the movie mission impossible.

Exfoliatins are the cause of "scalded skin syndrome" (SSS). This toxin was not identified until 1971 when it was first designated exfoliative toxin. It is produced in broth cultures and is recovered from the cell free supernatants as two types designated A and B.

The toxin causes a generalized exfoliation of the epidermis when injected into neonatal mice which is how it is assayed for biological activity. In humans, a dermatitis called Ritter disease, toxic epidermal necrolysis, exfoliative dermatitis, and bullous impetigo, are produced by the strains that yield this toxin. This group of conditions is collectively called scalded skin syndrome. It begins as an intense, tender erythema which is localized around the face and trunk and later spreads to the extremities. The epidermis is loosened producing large flaccid bullae. The epidermal layers separate and large sheets of skin peel away, exposing the underlying, sensitive dermal surfaces. A secondary desquamation ensues for several days followed by healing, without scarring in about two more weeks.

This toxin acts only on the cells of the granular layer of the epidermis where it disrupts desmosomes which bind adjacent cells causing a cleavage of the layers. The toxin is antigenic and results in antibody production which allows for recovery from the toxin attack but not the infection organisms. Human serum globulin does not contain enough antitoxin to be useful in therapy.

This toxin has the specific ability to disturb the adhesive forces between the cells of the stratum glanulosum which causes the characteristic bullae. It is potent, requiring only .2-.6 *u*m of the purified toxin to cause the skin separation. This small amount will easily fit on the tiny dust particles used in dust carrier weapons. It enables the direct entry into the underlying tissues of the skin to other organisms making it a particularly useful enhancing device for dermal attack.

[Authors note- In WW1, the use of gas masks and effective filters quickly rendered most chemical warfare agents obsolete on the battlefield and made their use only marginally effective for the next two years of war. The advent of a chemical (mustard gas) that was effective by skin contact alone produced a tremendous revolution in the effectiveness of chemical weapons and very nearly turned the tide in favor of the Germans who first used it.

By developing dermally effective biological ordnance that only requires a tiny skin contact to ultimately wound or kill an enemy is also as revolutionary. The physical barriers to inhalation and ingestion do not matter and it is impractical for an enemy to live in space suits permanently. This makes the exfoliant toxin, which is non lethal by itself, a very deadly weapon when combined with other toxin producing microorganisms. It will not matter that the body produces antibodies because the other bacteria and toxins take over the attack once this toxin has breached the primary dermal defenses.]

Pyrogenic Exotoxins were first isolated in 1979 as a protein that is pyrogenic, mitogenic for lymphocytes, and enhances the effects of certain endotoxins resulting in lethal shock and myocardial and liver damage. Three kinds were discovered and designated A, B, and C. The C toxin has been associated with toxic shock syndrome. These toxins produce a scarletiniform rash by inducing hypersensitivity (Kawasaki disease).

Coagulase enzyme enables S. aureus to clot blood plasma. By clotting the blood, a physical barrier is created preventing the bodies defenses from reaching the infection site (it also limits the rapid spread of the organism). It aids in protecting against intraleucocytic destruction by inhibiting phagocytosis and antagonizes the bactericidal of normal serum. These effects increase the virulence of the bacteria.

S. aureus coagulase exists in two forms - Free and Cell Bound. They are immunologically dissimilar and act differently. Free coagulase is a protein with four different antigenic types. It reacts with a plasma factor similar to prothrombin to yield a complex of coagulase and reacting factor that has a thrombin like enzymic activity which cleaves fibrinogen. This produces a fibrin clot.

Cell bound coagulase (clumping factor) is not released from the cell surface. This results in clotting occurring only on the surface of the cells as fibrin precipitates there. They clump as a group of cells when added to plasma and the fibers often are seen forming bridges from one cell to another.

Coagulase is not very toxic by itself when injected parenterally, but in large doses it produces extensive coagulation in the lungs resulting in rapid death. The use of either type of coagulase is recommended in combination inhalation weapons acting as an enhancement. The formation of clots protects the infective bacteria and provides a surrounding food source for both aerobic and anaerobic bacteria.

Coagulase protein is purified by ethanol precipitation followed by gel filtration on Sephandex G-200. It is heat labile, stable in acid pH with its isoelectric point at pH 5.3, is nondialysable and can be decomposed by proteolytic enzymes.

In vivo testing shows that staph coagulase has a very much higher activity than thrombin and produces almost complete defibrination. It also causes a rapid aggregation of blood platelets which aids in the coagulating activity.

Hyaluronidase consists of several enzymatically active components that attack and depolymerizes hyaluronic acid which is the ground substance of most human connective tissues. This enzyme is formed by most (90%+) strains of S. aureus. It is antigenically homogenous and enhances the initial invasiveness of the staph. The inflammation produced by the body is antagonistic to the spreading action of the hyaluronidase which slows its spread beyond the localized areas.

Staphylokinase is a substance that dissolves fibrin clots. This bacterial kinase produced by a large number of staphylococci acts on the plasma of many animals including dogs, guinea pigs, and rabbits.

Lipase's are produced by most staphylococci. Several lipid hydrolyzing enzymes have been identified that act on plasma, fats, and oils that accumulate on the surface of our bodies. The staph consume these and this is principally how they survive on our skin. The production of these enzymes is directly related to the organisms ability to form surface boils.

Nuclease is produced by 90%+ S aureus strains that has exo and endo nucleolytic properties that allow it to cleave either DNA or RNA.

Testing for pathogenicity is done in rabbits in which the test animal is injected with a small quantity of culture subcutaneously. Pathogenic strains produce localized abscesses and intravenous inoculation leads to either septicemia or pyaemia with multiple abscesses in the kidneys, lungs, or other organs.

[Many organisms produce a wide range of mutants when exposed to ultraviolet light during growth. Most of these do not produce toxins. A few will sometimes alter in their ability to infect (increase their virulence) and some will become more efficient toxin producers. This has been found to be the case with S. aureus.]

Protective Measures

You can inhibit growth of Staph with crystal violet at 1 part per 500,000 and brilliant green at 1 part in ten million. Antibiotics at one time were effective against staph species but most strains today, especially those found in hospitals, are antibiotic resistant. [These strains are easily recovered along with other disease causing organisms that can be resistant by simply walking hospital corridors and collecting air or floor dust samples and swabbing air filters.] Washing hands with antiseptics is also an important means of limiting infection spread by personal contact.

The staph strains become resistance to antibiotics by acquiring new genes from viruses (bacteriaphages) that specify the new resistance. Because of this ability, the organisms regularly acquire new resistance methods to each antibiotic they encounter and as a group they are more often drug resistant than all other types of bacteria. Multiple drug resistance is also quite common today and these strains that are also virulent make the most effective weapons. These drug resistant species usually are replaced over time by the less resistant ones so long as their is no intermittent exposure to the drugs in question.

Erythromycin and novobiocin are of some help with non hospital staph infections. Those strains originating in hospitals must use antibiotic sensitivity testing to determine which one, if any, might be useful. The staph will usually acquire resistance to the antibiotics in 7-10 days so they will likely not be useful for longer than that. Methicillin, oxacillin, cephalothin, and lincomycin have all been used for penicillinase producing strains. [Strains that are penicillin resistant produce the enzyme penicillinase that attacks penicillin.] Removing foreign bodies and draining the infection site are also important but may not always be effective.

Toxoids have been used and been found to not be effective. Usually the closed lesions must be drained as part of any treatment. The antigenic makeup of the Staph and the fact that many of its antigens (more than 30) are shared with other species is so complex and so broad that all the technical difficulties have largely precluded their possible use.

Phagocytosis is the most significant means that the body has to combat staph infections. Although there is an immune response to staph infections, there does not appear to be an improved bactericidal effect. Most of the toxins and enzymes produced by the bacteria serve as virulence components and these are immunogenic. Those staph strains that produce capsules are the most resistant to phagocytosis.

Of special interest is a substance called protein A, an antigen found on over 90% of the cell wall of the S. aureus. It provokes antiphagocytic and anticomplimentary effects, causes platelet injuries and hypersensitivity reactions in the host tissues.

Incorporation into Weapons

The production and recovery of the coagulase enzyme can be an invaluable weapon when combined with other organisms like the gas gangrene group and the other anaerobes. It is also produced in substantial volume by the staph organisms. This combined with the fact that both the staph and gas gangrene organisms can be recovered from the human body allows the production of this type of combination weapon in conditions of incarceration and allows for organism recovery and mass production without even leaving in home envoirenments.

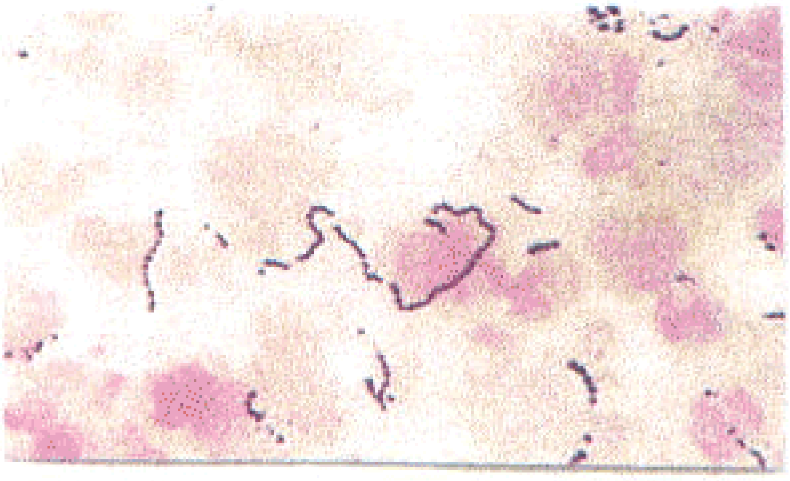
The coagulase enzyme, in combination with the exfoliatins, provides means of attacking and penetrating the skin tissues while preventing the body from being able to fight back. This makes it possible to conduct both aerobic and anaerobic attacks dermally. Toxin incorporation into the weapons in addition to the bacteria is critical since the ability to invade tissues by the staph organisms depends on the ability to bind to receptor sites that are usually already taken. Physical attack and injury to expose tissue with free binding sites is a pre-requisite to bacterial attack.

Adding the previously mentioned metal ions like magnesium or cobalt as well as other enhancing nutrients is sensible for the appropriate toxin or when using the organism in the weapon design.

To successfully invade skin, the bacteria must hydrolyze a wide range of skin materials and dissolve them allowing access to the underlying tissues. To do this requires protease's, lipase's, esterase's, and lyases along with many others we have already described. Once the skin is hydrolyzed and an organism is established and feeding in the skin and mucus membranes, the host defenses must be counteracted. The most successful single species at accomplishing this is the S aureus and it, and its toxins may be successfully incorporated into a wide range of biological ordnance with expected success.

[I spent years growing many organisms but gave little attention to S. species until writing this section. Setting aside all eloquent and technical language, I find it amazing that so many types of weapons can be produced simply by blowing your nose. It is also astounding how much power can be had by ordinary people simply by learning and comprehending the technically correct meanings of about 200-300 words of the English language. Knowledge really does translate to power.]

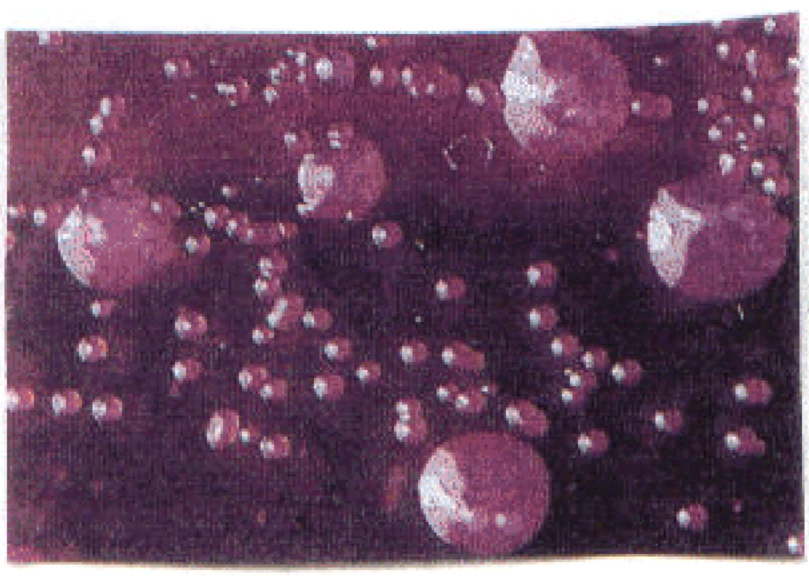
Long and short chains of streptococci stained gram positive with some pairs in a pus sample.



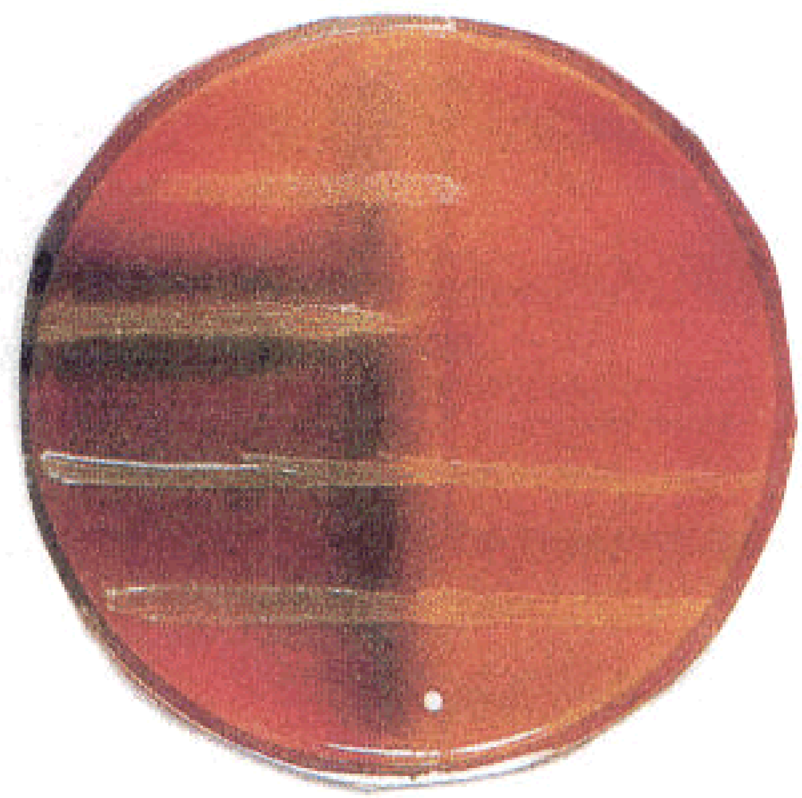
Alpha hemolytic streptococci. Colonies surrounded by a zone of gray-green discolored hemoglobin. (S. viridans)



Large colonies of E. coli surrounded by the small enterococci (S. faecalis) on a bile (10%) plate.



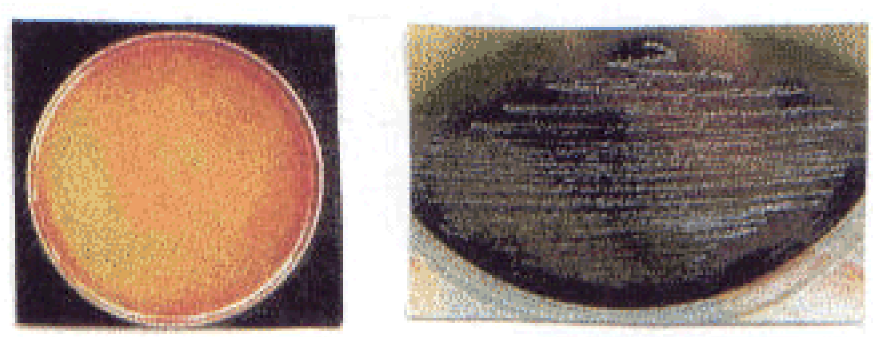
Blood agar with 10% bile on one half. Only two strains of strep grow on the half with the bile while all four grow on the blood.



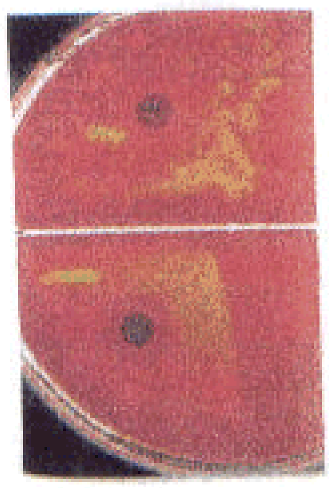
Tiny colonies of Streptococci growing right up to the neomycin disc while the S. aureus is inhibited by the antibiotic.



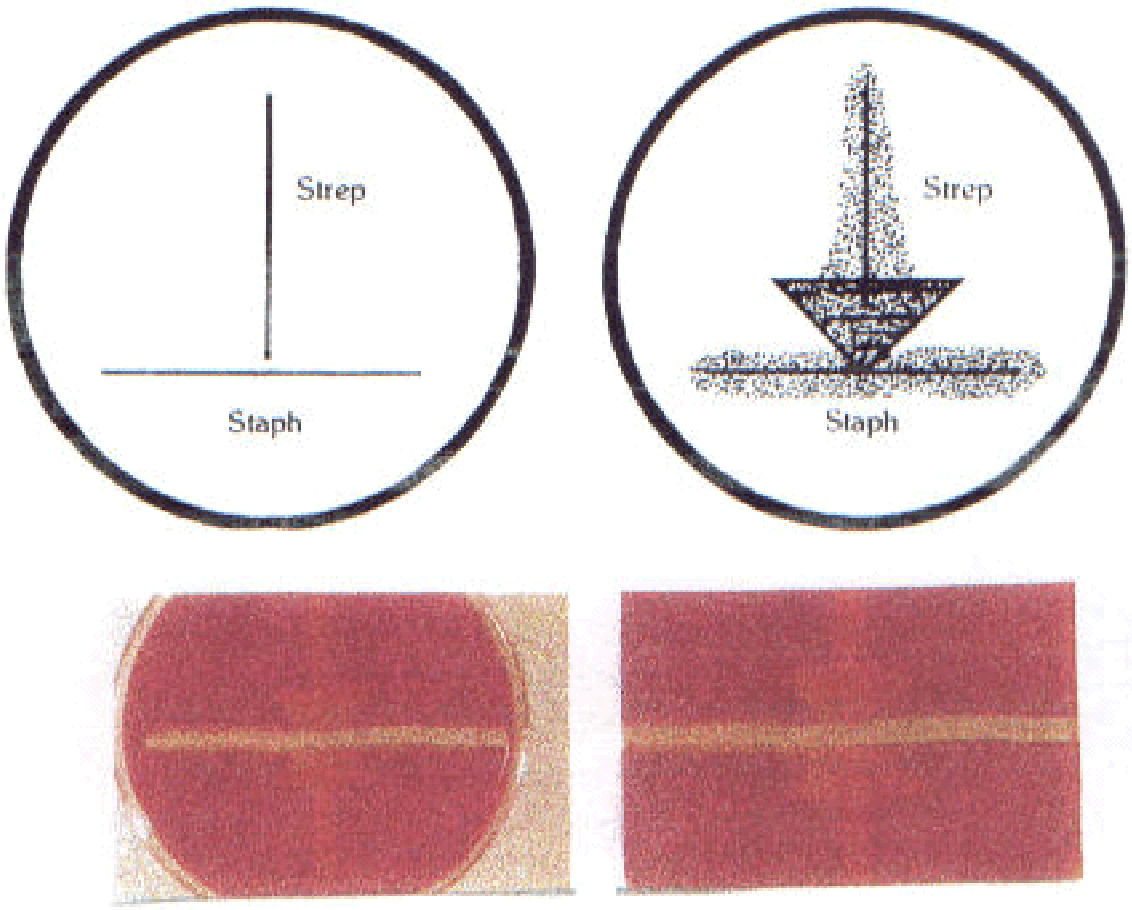
Green alpha hemolysis on blood agar by strep growth (left) Growth on bile-esculin agar on right. Note brown discoloration.



Blood agar biplate with colonies of beta hemolytic Group A strep. Intense reaction in the subsurface streaks showing the production of oxygen sensitive hemolysin



Staph-strep CAMP test using both organisms to produce arrowhead shaped combined hemolysis.



All members of the genus streptococci are facultative anaerobes. They ferment a number of substances with lactic acid as the principal end product. They are catalase and oxidase negative. Members of this group are easily killed in 30 minutes at 60 C in contrast to group D strep.

Optimal growth of the primary species S. pyogenes is 37 C with a pH of 7.4-7.6 on blood or blood products with the outside growth range of 22-42 C. Growth may be improved by reduced oxygen in the media or air. This is because the organisms produce hydrogen peroxide which can accumulate to lethal levels. Catalase which the organism cannot produce is present in the blood and thereby enhances aerobic growth. Thermal death point is about 54 C in half an hour. They survive for weeks to months in dust if out of sunlight or ultraviolet light. Culture plates must be stored at 3-5 C in blood broth or cooked meat in order to survive long on the shelf.

Most group A strep are beta hemolytic on sheep blood and adding a tiny amount (.05%) glucose may decrease this reaction. Sheeps blood is preferred because it inhibits Haemophilus haemolyticus which reacts very similarly to strep A organisms. Human blood is usually not used because it may contain antibodies and other inhibitory substances.

Streptococcus

History and Recovery from Nature

As a single genus, the streptococci have possibly caused more widespread disease and morbidity in man than any other bacteria except tuberculosis. In 1836, Bright first recognized a relationship between scarlet fever, acute glomerulonephritis, and chronic renal failure. Pasteur, Koch, and Neisser, while working on their germ theory of disease postulated that streptococci were the cause of puerpal sepsis when they observed chains of streptococci in the pus of a wound.. Ogsten defined the role of strep organisms in postsurgical wound infections, and in 1932, Coburn discovered the relationship between strep and rheumatic fever.

Man is the most susceptible of all animal species to streptococcus. When infection starts, the strep often invade neighboring tissues causing many types of disease. It is most often spread by spray droplets (sneezing, coughing), dust, and fomites (books, toys, etc.). Children are the most likely cause of organism spread, especially to other children at school.

Most streptococci are recovered from sputum, exudates, and excreta and survive for weeks in these fluids without a host. Serum coated swabs work best to recover organisms from tissues or fluids. Direct smears from pus and other infection sites is best.

Under the microscope, they are gram positive with oval or spherical cells about 1 um in diameter and arranged in pairs or chains with varying lengths. The cells divide by binary fission in parallel planes and many cells remain attached to each other resulting in the chains which vary in length that is determined by the firmness of the attachment of the cells. Some strains produce long chains while others may produce only one or two pairs of diplococci. Chains occur to the greatest extent in liquid media. Stains from agar and exudates may resemble staph aggregates or occur in single cells or pairs.

Strep do not form spores and are nonmotile except for a few strains. Their colonies are .5 mm in diameter on solid media with entire margins and are slightly granular. Cells from older cultures are often observed as being swollen to several times their normal size.

When solid culture media were developed near the end of the 19th century, it finally became possible to study the streptococci in the laboratory. Schottmuller first demonstrated the hemolytic reaction produced by strep on blood agar and Brown described the different hemolysis reactions, identifying them as alpha, beta, and gamma hemolysis. The beta reaction produces a clear zone of hemolysis on blood agar and these species are the cause of most strep disease in man. Strains that produce a narrow zone of partial hemolysis with greenish pigmentation are called alpha hemolytic. These strains do not produce a soluble hemolysin.

In the 1930's, five different antigenic groups of streptococci designated A, B, C, D, and E were identified based on the serological differences of their cell wall carbohydrates. Since then, the number of serological groups has risen to more than 20. The vast majority of human infections are caused by groups A, B, and D and these are the ones most useful in weapons designs. Some strains produce pigment in their colonies, mostly group B and D strains that may show rd and yellow.

Group	Species	Hemolysis	Human habitat	Diseases caused by
A	S. pyogenes	Beta	Pharynx skin	Erysipelas Acute pharyngitis Wound cellulitis Septicemia Rheumatic Fever Glomerulonephritis Rheumatic endocarditis
B	S. agalactae	Beta (Alpha)	Pharynx Vagina Stool Newborn-all	Peurpal sepsis Endocarditis Pneumonitis Neonatal infections
D	S. faecalis S. faecium S. durans S. bovis S. equinus	Gamma-reactive (Alpha) (Beta)	Bowel	Peritonitis Endocarditis Abscesses Wound infections
None	S. pneumonia (see next section)	Alpha	Pharynx, mouth Trachea	Lobar pneumonia Septicemia Otitis media Meningitis Endocarditis

The type of hemolysis produced on blood agar is the most helpful in telling the species apart in homemade labs. With a little experience a person will soon become accustomed to what the correct species is. The type of animal or human blood makes a difference in the reactions. Also the inclusion of reducing sugars will interfere with the results. Dextrose, fructose, galactose, and many pentoses suppress the lytic action of the streptococci on the blood cells, possibly by lowering the pH which inactivates the "streptolysin".

Group A streptococci are beta hemolytic and are the cause of many primary infections like acute pharyngitis, pyoderma, scarlet fever, erysipelas and cellulitis. It easily can cause complications such as rheumatic fever and endocarditis, valvulitis, and glomerulonephritis. By adding a small amount of the topical antibiotic "Neosporin" to the surface of the solid media in a streak after the inoculation, you can observe that no group A streptococci will grow near the streak while most other types will grow right up to it and sometimes in it. This is due to the bacitracin and neomycin present in the antibiotic preparation and it is also the choice used for many topical skin infections. Neomycin alone does not usually stop the strep but it does stop staph and allows for species separation in this way.

Group B streptococci are routinely recovered from vaginal cultures in the third trimester and are implicated in peurpal sepsis and most newborn infections. It is also often recovered in stool specimens and will normally grow right up and into the streak of Neosporin described for group A. Most group B strains are beta hemolytic. When grown under anaerobic conditions they sometimes display an orange or red-brown pigmentation.

Another method for identifying Group B strains is the "CAMP" test. A streak of staph that produces beta lysin is made on a blood agar as shown. A streak of suspected Group B strep is then made perpendicular to the staph. The plate is incubated aerobically and a combined zone of lysis is produced that assumes the shape of an arrowhead.

The group B strains are the most common cause of urinary tract infections and are also recovered from wounds, deep pelvic abscesses, and in septicemia and subacute endocarditis. The disease is caused by the many toxic enzymes produced by these organisms including hemolysins, camp factor, DNAse, hyaluronidase, protease, and neuraminidase all of which are serologically different from those produced by group A strains. The erythrogenic toxins are not produced by this group of strep.

Group B streptococci are bile esculin negative. This procedure will be described next for the group D strep.

Group D are often recovered from stool and sewage samples. These streptococci are divided into two groups -

> **Enterococci** which are penicillin resistant and require multiple antibiotics to stop. S. faecalis and S faecium are the species most commonly recovered and are often identified simply as enterococcus. These species grow in broth containing heart infusion and 6.5% salt (sodium chloride). The nonenterococci are inhibited by this concentration of salt.
>
> **Nonenterococci** include species of S. bovis and S. equinus are often called group D streptococci.

Group D streptococci may show alpha or beta hemolysis but are often nonhemolytic on blood agar. The same strain will also vary in its hemolytic properties depending on the type of animal blood used.

Group D can be differentiated from all other types of streptococci by their ability to hydrolyze esculin in the presence of bile. When 1-4% bile is added to the medium along with iron citrate .05% and esculin at .1%, most other bacteria are inhibited. The bacteria capable of growing in bile and at the same time able to hydrolyze esculin (Group D streptococci), will produce glucose and aglycone esculetin. When iron citrate is added to the medium the esculetin reacts with the iron to form a dark brown or black complex which diffuses into the medium and around the colonies.

With the bile-esculin and salt tolerance tests you can group the strep as -

1. Positive in both - growing in 6.5% salt and brown halo in bile are enterococci.

2. Negative in both - Are alpha hemolytic (viridans) streptococci.

3. Positive in bile and negative in salt broth are nonenterococci group D.

4. Withstands heating for 30 minutes at 60 C and negative in salt = S. viridans

S. viridans or viridans group are a large number of alpha hemolytic streptococci that resist classifying into a group by specific surface carbohydrates. These are generally found in the mouth and upper respiratory tract and include S. pneumonia which will be covered in the next section, S. milleri, and S. mutans.

S. mutans is interesting in that it produces a dextransucrase enzyme that yields an insoluble dextran polymer out of sucrose that we eat. This dental plaque that it produces on our teeth may contain up to 10 billion S. mutans per gram.

Streptococci produce long chains of tiny, gram positive cocci measuring .5-1 um in diameter. The cells may be elongated along the axis of the chain. They do not produce spores but several virulent strains produce capsules. Virulent strains are not motile and grow both aerobically and anaerobically. Older cultures tend to lose their gram positive staining. Cell division occurs in one plane only resulting in pairs or chains of organisms under the microscope.

Strep grow on most ordinary media but much better on blood containing media. Some pathogenic strains require extra enrichment to grow. The optimal growth temperature is 37 C and growth drops to near zero outside of 30-40 C. The pH must be 7.4-7.6. Growth is enhanced by adding .5% glucose to the medium.

S. pyogenes is almost always acquired from person to person contact and is usually not found outside a human host. The other species are widely distributed in man, most domestic animals, rodents, and many lower animals. Throat swabs account for most of the strep organisms recovered in medical practice. For recovery of strep, even from healthy individuals, blood or serum coated swabs work best. Most areas of the body will harbor one or more species of streptococcus.

The addition of crystal violet at one part per 500,000 to the blood media makes it selective for strep, especially when bile is added to solid media or 6.5% salt is added to broth.

Growth and Production

Strep grows both aerobically and anaerobically making this a good cross-check for isolating the correct or desired species. Anaerobic growth yields much smaller colonies (.5 *um* or less) and are pleomorphic in artificial cultures. Anaerobes after 48 hours on blood agar are smooth, low convex, and no alterations in the medium are seen (no hemolysis). In meat broth, a exceptionally foul odor may be produced.

Growth is enhanced in most strains by the addition of 10% CO2 to the medium or air. The addition of phosphate buffer and glucose to the medium also enhances growth, especially on infusion mediums with fresh blood. Group A strains are stimulated by the addition of peptides to the medium.

Streptococci produce two hemolysins, streptolysin O which is Oxygen-labile (which means it is inactivated by oxygen) and is antigenic, and streptolysin S which is oxygen stable but non-antigenic. Each of these hemolysins produce a complete clearing of the blood agar around the colonies. Strep organisms grow both aerobically and anaerobically and are incubated on top of solid media and also inside the media in agar stabs in order to compare and observe the hemolysis reactions under both conditions.

Group A streptococci measure .6-1 *um* in diameter, are spheroid to ovoid, and most form long chains in liquid media, a phenomena which is enhanced by adding type specific antibody to the growth medium. They usually stain gram positive but may not absorb the stain or stain negative as the culture ages.

Group A S. pyogenes is not normally found on the skin of humans as the lipids in skin (oleic acid) are bactericidal to it. If it does reach the underlying tissues via even a tiny injury it causes a variety of disease.

Some strains produce a capsule hyaluronic acid which is present during the first 2-4 hours of growth but most strains also produce hyaluronidase which destroys the acid as the culture ages. Those strains with this capsule possess superior virulence over other strains.

When growing in cultures, both plate streaking and pouring into deep broth or gel/agar mixes are used. After 18-24 hours on blood agar, S pyogenes colonies are domed, grayish to opalescent, semi-transparent, low convex discs, and about .5-1 um in diameter. Their zone of complete hemolysis is several times the diameter of the colonies. The zone may be enhanced in submerged inoculation and growth or in reduced oxygen atmosphere. Virulent strains most effective for weapons give a matt type or mucoid colony while avirulent strains tend to produce glossy colonies.

Group A strep (about 95%) are very sensitive to bacitracin and streaking a small amount of Neosporin on a plate strongly inhibits the growth of group A strep nearby. Staph also grow and produce similar hemolysis and are distinguished by the catalase test - strep are catalase negative, and the colony morphology is different. They are also very sensitive to antiseptic dyes.

A bacteriophage that infects strep organisms determines if the strep will produce an erythrogenic toxin. There is also a phage that can direct type C to produce a muralysin (N-acetylmuramyl L-alanine amidase) that is released on cell lysis that not only lyses blood cells but strep group A cells as well.

Group A streptococci produce three surface proteins designated M, T, and R. For purposes of weapons development, the M protein causes the strep to be highly resistant to phagocytosis and this confers superior infective capability if the specific antibodies are not present. Serotyping with antisera for M protein is required to identify those strains and will not be covered here.

In a nonimmune host, injection of group A streptococci onto the surface of the pharynx or under the skin will produce disease. Those strains with the M protein resist phagocytosis and proliferate. They can remain localized or invade the circulatory system and reach other tissues causing serious infection and complications. The host then produces antibodies and the organisms are usually contained in a local site or eradicated. Long lasting immunity often occurs with the pharyngeal infections. Once ingested by the neutrophils or monocytes, the strep organism is rapidly killed and complete disintegration occurs in 1-4 hours with the exception of the strep cell wall which is resistant to breakdown enzymes and may persist in cells or tissues indefinitely.

Many group A strep strains have improved virulence for establishing respiratory infections. It has been observed that pharyngeal or cutaneous strains may bind selectively to epithelial cells at these sites.

Group B streptococci (mainly S. agalactiae) are beta hemolytic and have the same morphology as the other beta hemolytic species. They tend to grow in short chains or as diplococci in liquid media such as broth's which is helpful in distinguishing them.

A hemolysin that is not SLO is produced and may cause a double zone of hemolysis on rabbit blood agar after inoculation, a short incubation, and then refrigeration. Colonies usually grow to more than 2 mm in diameter and are usually large and mucoid producing a small zone of beta hemolysis. Almost all strains produce cartenoids which give them a red, orange or yellow pigment on different medias. Adding glucose to the medium suppresses this pigment production.

Group B streptococci can hydrolyze sodium hippurate, most grow in 6.5% sodium chloride, and some grow in 40% bile. A few strains may be bacitracin sensitive and none hydrolyze esculin.

Group B are commonly found in the pharynx, GI tract, and in the vagina of 5-1`0% of pregnant women. It is found to colonize the urethra of male sex partners (and the rectum) and is therefore sexually transmitted. They cause wound infection, puerperal infection, neonatal septicemia, and meningitis. Most of these occur after obstetric complications and caused nearly 100% mortality prior to the introduction of antiseptics and antibiotics. Mortality today is still about 50% and even higher if within 10 days of giving birth. Most strains are sensitive to penicillin G, erythromycin, chloramphenical, cephalosporins, lincomycin, and clindamycin.

These organisms also are the principal cause of mastitis in cattle and the presence of the organisms in milk requires pasteurization for it to be safe.

Group C streptococci include the species S. equisimillis, S. zooepidemicus, S. equi, and S. dysgalactiae. They have colony morphology similar to type A organisms and are beta hemolytic except for S. dysgalctiae which produces alpha hemolysis only. S equisimillis produces SLO, streptokinase, and other toxins which are excreted outside the cell and which produce antibody responses during infection. Most of these species do not produce disease in man but are found in the upper respiratory tract of many domesticated animals.

Group D streptococci typically grow as short chains or diplococci. A few motile strains are seen. This group is divided into the -

Enterococci	Nonenterococci
S. faecalis	S. equinius
S. faecium	S. bovis
S. durans	

The enterococci are capable of growing in 6.5% sodium chloride while the nonenterococci cannot. Group D are isolated and distinguished from other species by their ability to grow at 45 C and survive at 60 C. They grow in the presence of 40% bile and hydrolyze esculin. All produce alpha or gamma reactions on blood agar except for one strain of S. faecalis (zymogenes) which is beta hemolytic.

The strains can be separated by their following biochemical reactions.

	S. faecalis	S. faecium	S. durans	S. bovis	S. equinus
Bile esculin	+	+	+	+	+
6.5% NaCl	+	+	+	-	-
Sorbitol	+	-	-	+/-	-
Mannitol	+	+	-	+/-	-
Lactose	+	+	+	+	-
Starch	-	-	-	+	+

Group D often inhabit the upper GI, genitourinary, and upper respiratory systems of man and animals. A majority of Strep infections are caused by invasion of these normal flora on and in our bodies. These infections include septicemia, endocarditis, wound infection with gangrene, abscesses, appendicitis, and urinary and biliary tract infections. Elderly and heart disease patients appear the most susceptible. When group D strep are recovered from the blood, they often are also responsible for occult tumors.

Most of these strains are resistant to penicillin G and penicillinase-resistant penicillins. Penicillin combined with aminoglycosides like gentamycin or streptomycin are often effective. Vancomycin and erythromycin are also used in these infections.

S. faecalis have smaller colonies of .5-1 *u*m diameter and are usually magenta colored. They can grow at temperatures as low as 10-15 C. All the enterococci ferment mannitol with gas production which easily differentiates them from other strep species.

Viridans streptococci contain a large number of alpha reacting organisms that cannot be classified by the normal serotyping. This group is almost always found in the mouth or upper respiratory tract and include S. pneumonia (next section), S. mitior, S. milleri, S sanguis, and S. mutans. This last strain produces as many as 10-100 billion cells per gram of dental plaque.

They invade the bloodstream following dental work, invasive internal examinations, and even during chewing if skin injuries occur. Cellulitis, wound infection, meningitis, sinusitis, endocarditis, and biliary or intraabdominal infections are caused as well as liver or brain abscesses (S. milleri). S sanguis is the most frequent cause of endocarditis.

	S. mutans	S. mitis	S. intermedius	S. anginosis	S. uberis
Mannitol	+	-	-	-	+
Lactose	+	+	+	-	+
Sucrose	+	+	+	+	+
Raffinose	+	-	+/-	-	+
Inulin	+	-	-	-	+/-
Esculin	+	-	+	+/-	+
Hippurate	-	-	-	-	+/-
Arginine	-	+/-	+/-	+/-	+/-
Litmus	+	+	+	+	+
40% bile	+/-	+/-	+/-	+/-	+

Their colonies tend to be smaller and more convex than group A strep and their hemolysis often has a greenish tinge with a thin outer rim of complete lysis after being stored in the refrigerator. Heated blood agar produces the best green pigment.

They are almost always recovered from throat swabs and sputum. Thermal death point is app. 55 C in half an hour.

Toxicity and Harm

Alpha hemolysis refers to a type of incomplete hemolysis where red blood cells surrounding colonies are partially damaged but not lysed as in beta hemolytic reactions. Their is a characteristic "greening" of the medium caused by hemoglobin which is oxidized to biliverdinlike compounds which leak out of the cell. It has a slimy consistency surrounding the colony. The alpha hemolytic streptococci are often referred to as S viridans which is not a single species but refers to a group of strep with no serological specificity. These bacteria are often called "green strep" and are a frequent cause of subacute bacterial endocarditis. By examining the culture media under the microscope, you can see that the cells are not intact but are not completely destroyed as they are in Beta hemolysis. In alpha hemolysis, the red cell outlines are clearly seen and can often be distinguished with the naked eye. In Beta hemolysis, their are no red cell outlines remaining.

Beta hemolysis as already stated involves the complete destruction of the blood cells so that no part, including the cell wall, remains intact. There is a clearly transparent zone usually extending several times beyond the diameter of the colony.

Gamma (No hemolysis) produced by most S. faecalis and almost all non-pathogenic strains on blood agar.

For best hemolysis test results, horse or rabbit blood should be used. Sheep's blood is OK for most strains and human blood should not be used.

As a general rule, those colonies that produce mucoid and matt colonies are considered to be virulent strains while the glossy colonies tend to contain little of the cell wall proteins that cause the mucoid appearance and are considered to be mostly nonvirulent. [Recovery of mucoid strains with hemolytic abilities make good weapons.]

Strep organisms are capable of infecting every organ or tissue of the body in a manner similar to the staph organisms already described. Before antibiotics, they produced very high mortality rates. Almost every child by the age of 10 has had a bout with at least type A strep. Immunologic surveys of school age children show that most have had an infection in the preceding 3-6 months. The upper respiratory tract infections occur most frequently during the winter months and are transmitted primarily by respiratory droplets during coughing and sneezing.

When foodstuffs are contaminated major epidemics have occurred but the pasteurization of these products has all but eliminated this threat. In infections, the strep are best recovered early and may often be the only organism recovered. Later in infections, there may be a swarm of other organisms for whom the strep has opened the door. Strep is in fact a good door opener for a variety of biological ordnance as well.

The most common infections are -

Epidemic sore throat - caused by group A strep, particularly S pyogenes. Epidemics occurred in the US and England in the early 1900's. Intense local hyperemia occurs, sometimes with a grayish exudate and enlargement of the cervical lymph nodes and with fever. The extension occasionally extends into the lungs with pneumonia followed by fatal septicemia. The sequelae of strep sore throat can extend the infection into adjacent areas such as the sinuses and middle ear resulting in chronic infections.

Scarlet Fever - caused by an erythrogenic toxin produced by some strains of group A strep. This toxin, called "Dicks toxin", usually initiates an immune response with antibodies produced. A small amount of this toxin (.2 ml) is used and injected in a small amount intradermally producing a rash around the injection site in 24 hours unless the individual carries sufficient antibodies to neutralize the toxin. A small amount can be heated to destroy the toxin and then be used as a control. This establishes the immune status of the host.

The rash is an erythema that blanches on pressure and initially involves the trunk and neck, and then spreads to the extremities. It is usually spread from a pharyngeal infection in the upper respiratory tract, most often on the tonsils, and may involve several episodes due to the three different erythrogenic (pyrogenic) toxins produced by the strep organisms.

Long lasting immunity follows recovery from this infection and extends to other types of strep infections involving the M antigen. Penicillin is used to prevent extension of the infection - rheumatic fever.

Subacute Bacterial Endocarditis (S.B.E.) - can be caused by any organism that enters the circulatory system and establishes itself on the heart valves. The S. viridans group are the primary infective agents usually originating from a tooth extraction which produces bacteremia. A bacteria or fungi implants itself onto the endocardial surface of the heart causing fever, weight loss, anemia, heart murmur, splenomegaly and peripheral embolization. Once established on the heart valves, the bacteria, usually attacked and destroyed in the bloodstream by phagocytic cells, now find protective covering inside the lesions they produce. They constantly shed more strep organisms from the lesion with resulting and continuous bacteremia. Despite this shedding they do not often establish themselves at other sites indicating low virulence or high immune response. Without treatment with antibiotics to remove the infective bacteria, the disease is always fatal.

Impetigo (pyoderma) occurs when the skin is colonized first followed by the pharynx. It occurs mainly in temperate climates during the late summer and early fall and resembles chickenpox. It starts with a vesicular rash that quickly becomes pustular. Later, the lesions become covered with a thick crust and the strep are found in the fluid beneath the crust, usually in pure culture. Staph soon infects the lesions as well but add little to the pathogenesis. It is normally caused by group A beta hemolytic strep and is considered endemic in the southern US and the tropics.

Pyoderma is usually spread by close contact where people live in crowded and unsanitary conditions. The strep colonizes the skin in tiny breaks such as mosquito bites or scabies infections where they proliferate.

Rheumatic Fever - the most serious strep infection caused mainly by S. pyogenes (Group A). Streptolysin O toxin antibodies are usually produced by the host but does not seem to render the patient immune. The detection of the antibodies aids in diagnosis by doctors.

It only occurs following respiratory infection, usually pharyngitis, by strep group A. Symptoms may include arthritis- especially in older people, carditis in younger people, chorea, erythema marginatum, and subcutaneous nodules. These symptoms occur within 2-3 weeks after the start of the infection to as long as 6 months later. Carditis involves connective tissue degeneration with damaged heart valves and myocardial lesions called "Aschoff nodules".

The disease begins as an acute infection of the nasopharynx. A quiescent period occurs followed by electrocardiographic changes in 18-19 days signaling the onset of rheumatic fever. Throat cultures are often negative by the time the onset of this final stage occurs. Attacks recur with reinfection of strep organisms. By the time the rheumatic attack occurs, antibiotics are ineffective. Antibiotic therapy to prevent repeat episodes often lasts as long as five years.

Rheumatic fever may occur in up to 3% of all individuals in pharyngeal epidemics. The mortality associated with rheumatic fever is linked to the development of rheumatic valvular heart disease which causes 15,000 + deaths per year in the US (1980). Most of the heart disease is caused by the strep exotoxins and direct invasion of the heart muscles and valves.

Skin Diseases - such as impetigo, erysipelas, and carbuncles result from a variety of strep infections. Group B, C, D, and G are frequently recovered from skin lesions. Group A causes all the above and gas gangrene when involved in wounds.

Impetigo is a very superficial skin infection that has crusting, amber lesions that may begin as small vescicles. The early lesion is usually strep but soon staph may superinfect with complications. Cellulitis with local endopathy and lymphangitis may occur during deeper invasion with systemic symptoms like chills, fever and malaise. If it invades the muscle it can spread to cause gangrene in patients with peripheral vascular disease or diabetes. It tends to spread rapidly via the lymphatic system and generalizes into septicemia. All groups of strep may become involved as well as anaerobic growth. It is a common infection in hot, humid climates with crowded living conditions. Most of these could be prevented with good skin hygiene.

Erysipelas is a skin infection usually occurring on the face and invading the subcutaneous tissues. It produces edema, erythema, and induration which usually has a distinct border. Some individuals suffer repeat episodes, often in the same site. It is more serious than pyoderma with the strep organisms present in the lesions in enormous numbers in the periphery of the inflamed area. The strep occur in the lymph spaces of the skin in very large numbers in advance of the lesion (by 2-3 cm.).

Peurpal fever (sepsis) - also called childbed fever, develops when streptococci enter the uterus after delivery. This disease killed thousands of women before the advent of germ theory, disinfectants used in delivery instruments, and antibiotics. Today it is rare. The strep may be part of the normal vaginal flora or be introduced during delivery, obtained from the attending nurse or doctor.

Symptoms include fever, chills, facial flushing, abdominal distension with pelvic tenderness, and vaginal discharge. Group A strep are usually isolated form the lochia or blood. Even with antibiotics, recovery may be long and full of complications.

Sinusitis is an inflammation of the membrane lining the air filled cavities in the bones surrounding the nose by group A strep which usually originates from the throat. The sinuses between the eyes and in the cheekbones are also commonly affected. The infection spreads as mucus drains down the narrow passages from the upper sinuses into the nose.

Infection often follows a tooth abscess, jumping into water feet first allowing water into the deep sinuses, and almost always following a cold. Recurrence becomes more likely with each cold. Symptoms include stuffiness, fullness, fever, throbbing aches, loss of sense of smell, and a pus which discharges and from which the organisms can oftem be recovered.

Tonsillitis is a strep infection of the tonsils which often protect the upper respiratory tract from invading organisms. Symptoms include sore throat and diffuculty in swallowing with the tissues visibly inflamed. Fever ,earache, headache, and enlarged and tender glands in the neck with unpleasant smelling breath also occur. If pus forms on the tonsils, they have been infected with the organisms they are intended to fight and often must be removed. Removal of the tonsils does not offer protection against strep infections already established elsewhere in the upper respiratory tract.

Pharyngitis is usually caused by group A strep and when infected with a virus that causes production of a toxin it may become scarlet fever. Before antibiotics, this often led to suppurative complications like tonsillar abscess, otitis, septicemia, mastoiditis, and osteomyelitis as the organism spread unchecked.

Symptoms may or may not include sore throat, fever, chills, headache, malaise, nausea, and vomiting. Abdominal pain in children may be confused for appendicitis. The pharynx may be red with grayish yellow exudates which bleed when swabbed for culture, or they may only be mildly erythematous. Leukocytosis and anterior cervical adenopathy are commonly observed. Mononucleosis, diphtheria, herpes simplex, and many other respiratory viruses may appear identical and indistinguishable from the strep infection.

Pharyngal infection may confer lifelong immunity if not aborted early by the use of antibiotics. The S. pyogenes from these types of epidemics make the most virulent organisms for toxin production and incorporation into weapons.

Laryngitis is an inflammation of the larynx (voice box) resulting in a limited ability to talk (hoarseness). There is usually pain and diffuculty swallowing with a dry irritating cough. Sputum may be coughed up and will usually contain the infective organisms.

Pneumonia is a respiratory inflammation often involving many organisms and mucus production. It is the sixth largest cause of death (1989) and can become life threatening without the use of antibiotics. It is most common in male infants and elderly with reduced immunity to infection. There are two main types -

1) Lobar pneumonia where one lobe of one lung is initially infected.

2) Broncopneumonia where the infection starts in the bronchi and bronchioles and spreads to produce patches of infected tissue in one or both lungs.

Symptoms include fever, chills, shortness of breath, and a cough that yields a yellow-green sputum and occasionally blood with the infective organisms. If the membrane lining the lungs and chest cavity are also infected there will be considerable pain while breathing.

Glomerulonephritis (AGN) is a complication of group A skin or pharyngeal infections. It often follows pyoderma within 10 days to two weeks. Only certain strains cause this infection which most often afflicts children. It is characterized by acute onset of edema, oliguria, fluid retention, hypertension, headaches, nausea, congestive heart failure, nephritis and seizures. The urine turns dark or smoky, with various cells and serum as part of the fluid. The symptoms follow the initial infection in about 1-3 weeks. The granules in subepithelial deposits caused by the inflammatory response and accumulated cell and toxin components gives this condition its name.

When injected by IV in rabbits, the kidneys show lesions from strains producing this disease. The DNAse and hyaluronidase toxins are most often associated with the effects of this disease.

Meningitis is an inflammation of the three membranes (meninges) that cover the brain and spinal cord. It is a life threatening infection when strep reach and colonize the meninges from the bloodstream. The symptoms include fever, severe headache, nausea, vomiting, light sensitivity, and a stiff neck. They can develop in a few hours and are sometimes followed by drowsiness and loss of consciousness. In about 50% of the cases a red, blotchy skin rash occurs. A spinal tap is used to recover organisms and confirm the infection. If strep is recovered, it is considered a major medical emergency and massive amounts of antibiotics are given. Even if the patient recovers, brain damage is often the result.

Streptolysin O (SLO) toxin is produced by most group A and many group C and G streptococci. SLO and streptolysin S in combination are responsible for the complete beta-hemolytic reaction on blood agar. SLO is inactivated by oxygen but this can be reversed with reducing agents like cysteine or beta-mercaptoethanol. It is irreversibly inactivated by cholesterol. Strep organisms must be grown inside of serum free broth since the toxin is oxygen sensitive and does not survive when produced by surface colonies exposed to oxygen.

SLO is toxic to red and white blood cells and myocardial cells in tissue cultures. It can cause cardiac death on IV injection in animals in small doses. This toxin is a potent antigen in pharyngeal and systemic infections with antibody response occurring in 10-14 days in a first infection and sooner in repeat infections. Immune responses following skin infections are much smaller. Attack with SLO results in fever, frequent and deep respiration, diarrhea, and general depression. A temperature drop below 36 C and rapid drop of blood pressure are signs of imminent death.

SLO is produced in serum free broth's. It is considered to be present in the most virulent strains since it is capable of killing a wide variety of cells and is specifically cardiotoxic. It is considered thermo-labile, nondialyzable, and antigenic and may exist in either an active hemolytic, or inactive nonhemolytic form. Its greatest hemolytic activity occurs at a pH of 6.5 and temperature of 38 C and falls off rapidly on both sides of these figures. Individuals with high cholesterol combined with other lipids in the bloodstream are able to inhibit the effects of SLO somewhat. Caffeine and amphetamines decrease the hemolysis while narcotic drugs increase its effects.

SLO attacks erythrocytes, leucocytes, blood platelets, lysosomes, and human liver, kidney, and heart cells. It contributes to the formation of heart lesions in rheumatic fever. In tissue cultures it apparently cannot do all these actions on the specific cells alone and requires the presence and additional action of other strep toxins or the organism to accomplish its destruction. When injected, it can act on the heart directly by itself making it a direct potential inhalation weapon. Only the addition of antiserotonin compounds provided any degree of protection against this type of direct attack. The use of SLO as a direct attack agent seems to stimulate the production of protective antibodies by the targets immune system that end up attacking the hosts tissues.

Mice are about 30 times as susceptible to SLO as rabbits and guinea pigs. Venous congestion of the lung, liver, spleen, and heart are observed as well as intravascular hemolysis after IV injection. It is not lethal by the intraperitoneal route. SLO is universal in its effects on mammal heart tissue and its high pharmacological potency is well established. It causes clear damage to the contractile and pacemaker cardiac tissues of the heart and at high doses it interferes with the cardiac cycle so extensively that it caused death in test animals in 2-4 seconds after injection which is the amount of time it took to reach the heart. It also contributes to the formation of lesions on heart and liver tissues as well as scar tissue.

Media used to grow SLO is enhanced by the addition of cysteine (which also acts as a reducing agent to reactivate it) and ascorbic acid. As the medium accumulates lactic acid, it must be neutralized with baking soda or other buffers to maintain production and prevent destruction of the toxin. The peak of SLO synthesis occurs at 6-12 hours after inoculation in the media. Strains from healthy individuals do not usually produce good yields of SLO while strains directly from the infection sites of human hosts tend to produce very good yields. In some strep species, a proteinase enzyme is produced that attacks and destroys SLO so it is important to either dilute or separate these components early in the process Freeze drying will also inhibit this effect until ready for use.

SLO has been concentrated by fractionation with ammonium sulfate at 85% concentration, and elution from calcium phosphate gel. This is followed by adsorption and elution from alumina. These steps resulted in near purity.

Continuous flow electrophoresis and calcium phosphate column chromatography has also been successful and also recovered the DNAse as well in the same fraction.

Concentrated toxin has been commercially achieved with potency of 250,000 hemolytic units per mg. As little as 5-50 units are all that is necessary to cause cardiac arrest in most small test animals. Translated to humans, about 1,000 lethal cardiac doses are possible per mg. of this concentration of toxin. One milligram will easily fit on a near invisible dust particle.

The toxin also produces skin necrosis with the formation of yellow lesions in about six hours. Where the toxin diffuses into the surrounding tissues, it causes skeletal muscle destruction as well.

Streptolysin S (SLS) is an oxygen stable, highly soluble, non antigenic toxin by that can be extracted in quantity from strep cells with egg albumin, RNA or detergents added during production. It produces hemolysis in red and white blood cells and also bacterial protoplasts by direct cell to cell contact. It produces the surface hemolysis seen on blood agar and those strains lacking it may appear nonhemolytic in surface growth but hemolytic in subsurface growth. Over 95% of group A, C, and G strains produce SLS.

SLS is produced is produced only in media containing serum which is where the "S" designation came from. High potency filtrates are produced using yeast nucleic acids (RNA) as part of the medium. This induces formation of large volumes of the SLS in growing cultures even without the blood. By shaking the cultures in human or horse serum, and then washing them, it was found that SLS could be recovered from the fluid as often as five times in a row in equal concentrations. It is known that the serum solubulizes the SLS allowing it to be recovered in the filtrate. In fact the serum is required for its solubulization and recovery. Adding egg albumin also aids in SLS formation and recovery and should be included in the serum.

The RNA preparations of not only yeast, but wheat germ, liver, spleen and muscle cells, tobacco leaves, and kidney cells (at .8%) were all effective in inducing SLS formation in strep group A. Detergents effective in extracting the toxin while incubating include commercial octylphenols, palmitates, and stearates. The addition of magnesium ions and/or glucose (.005%) during incubation enhances hemolysis effect and diffusion into the medium.

Use of the detergents, glucose, and egg albumin in combined strep and toxin ordnance is recommended. The same strep organisms can be used repeatedly for toxin production as long as the added RNA extract, an energy source, glucose, magnesium ions, and sulhydryl compounds are present. This means the addition of maltose and cysteine. By adding thioglycolic acid, glutathione, and thiomalic acid it was found that yields could be increased five fold.

Most enzyme poisons such as disinfectants severely limit toxin production.

Horse and dog serum produces the highest yields of SLS while humans and monkey serum yield modest amounts and rabbit or guinea pig serum produces only small amounts.

Human blood serum contains lipids that have a modest inhibitory effect on SLS but the production of antibodies to the toxin provides no protection whatsoever to the hemolytic effect. There is a period of latency before the lysis of erythrocytes and leucocytes begins. IV injection in animals results in massive intravascular hemolysis accompanied by hemoglobinuria. It destroys platelets, lysosomes, and other subcellular structures. It also degenerates the epithelial cells of the kidneys.

SLS is highly concentrated by precipitation with ammonia and fractionation with ethanol resulting in hemolytic potency of 1 part per 100 million. A number of enzymes inactivate SLS such as amylase and intestine nucleophosphatase. The toxin is stable in a dry state but thermo-labile in aqueous solution.

It has been purified by initial precipitation with 25% methanol at 0 C followed by a second precipitation at pH 1.6 and then adjusted to .165 from water. The hemolysin is unstable in water and lose most of their activity at 25 C in a few hours. They are very stable in a dry state. Adding potassium ions to water solutions afford considerable protection to the toxins resulting in virtually no potency loss over a wide pH range.

SLO and SLS are produced only by type A, C, and G streptococci. Similar hemolysins are produced by type B. SLS is produced mainly in aerobic cultures while SLO is produced mainly anaerobically.

Streptococcal Leukocidin is recovered in culture filtrates and although different, it may be identical in action to SLO. Other intracellular hemolysins have been recovered from filtrates that are lethal, and in some cases specifically attack cancer tumors.

Erythrogenic Toxin is produced by most group A strep when, like C. diphtheria, they become infected with a particular virus (bacteriophage). These toxins are responsible for the rash seen in scarlet fever, reticuloendothelial blockade, act as a mitogen, produces myocardial and hepatic necrosis in rabbits, and decreases the hosts ability to synthesize antibodies at the T lymphocyte level. There are at least three different types of this toxin that are all heat-labile but are stable in acid, alkali, and pepsin. It produces the local erythema when injected into test animals and people.

The type C toxin is known to cause increased permeability of the blood brain barrier to endotoxin and bacteria. It exerts a pyretic effect by acting directly on the hypothalamus.

The toxin is precipitatable by trichloroacetic acid or alcohols and is present in high potency. It is also purified by electrophoresis recrystallization.

DNAses are extracellular enzymes that breakdown DNA (types A and C) and RNA (types B and D). This breakdown provides additional food for the streptococci. They require extra calcium and magnesium for peak activity. The antibody titers to the DNAse B are used in diagnosing pharyngeal and skin infections. They are produced by all strains of streptococci, are thermo-labile, and induce antibody formation. They are toxic to leucocytes.

Streptokinase is an enzyme (fibrinolysin) that acts as a catalyst for converting plasminogen to plasmin which leads to the rapid lysis of fibrin and protein hydrolysis in humans. This means that it destroys blood clots. There are believed to be several different kinds of streptokinase and all are inactivated by the action of trypsin. This is an effective weapon to be used in piercing attacks (pungi sticks) where profuse bleeding can be induced and not be stopped.

Streptococcal hyaluronidase hydrolyzes hyaluronic acid in animal tissues and in the capsules surrounding strep cells.

A proteinase is produced as the pH falls in the surrounding tissues or media. NADase, ATPase, phosphatase, esterase's, amylase, N-acetylglucosaminidase, neuraminidase, lipoproteinase, and a cardiohepatic toxin are all produced in small amounts by the streptococci and assist in breaking down the adjacent tissues for food as well as improving the invasiveness of the organism. The neuraminidase enzyme is the same one that enables the C. diphtheria to gain an infection foothold in its infectious cycle in the throat. This commends the use of these toxins and bacteria in combination weapons with diphtheria.

Subcutaneous injection into test animals of group A strep colonies produces local inflammatory and suppurative lesions, while intravenous injection results in septicemia with formation of multiple pyaemic abscesses if the animal survives. The most effective organism are the group A strep with high M protein content on their cell walls which enables them to establish themselves onto host tissues. The M proteins can be seen in the colonies producing matt or mucoid colonies and are low or not present in the glossy colonies.

The virulence of group A in mice is related to the production of capsules made up of hyaluronic acid.

Protective Measures

Pasteurization is used to kill streptococci in milk, cheese, and other human foods. This is done by heating at 60 C for 30 minutes. Most of the pathogenic strep are sensitive to sulfonamides and other antibiotics. Group A is considered sensitive to penicillin and erythromycin. Most of the diseases respond well to antibiotic treatment but some like rheumatic fever return at repeated intervals and require prolonged antibiotic treatment in small doses.

Disinfectants with free chlorine kills most strep species that contaminate furniture, surgical instruments and other objects. Gargling with antiseptics is also commonly used and is effective against many strep organisms for up to six hours. Antibacterial soaps and topical ointments are used to control strep populations and spread across tissues and to other individuals in epidemics.

Antibiotic therapy is usually intense and widespread to prevent minor strep infections from becoming major and life threatening. Surgical debridement and even amputation may become necessary when strep spreads to underlying tissues.

Incorporation into Weapons

Strep is best used as a door opener for the more deadly strains of bacteria. The strep can establish itself early but is usually poor in its ability to compete for surface sites with other bacteria. Its toxins help in breaking down adjacent tissues and resisting the bodies initial immunity and should be produced and used with the bacteria in combination weapons. It is easily recovered and mass produced and its toxins cover a wide range of activity. It is responsible for the widest range of disease among all the bacteria and make excellent enhancement (piggy back) weapons.

If the organism is to be used in the weapon, it should be a *B*-hemolytic strain since these have been well established as being the most virulent of this genus. Their toxins produce the most varied and potent diseases. Alpha hemolytic strains are generally the cause of secondary infections and rarely cause disease without help. All the toxins should be used in weapons together since it is these toxin combinations working together that enable invasion of the target and cause disease.

Use of streptolysin O toxin is useful in causing heart attacks, especially in those targets with established heart problems. Its use is nearly impossible to identify as a weapon and is for all practical purposes, indistinguishable from natural causes.

The erythrogenic toxins produce an erythematous rash (scarlet fever) when applied in large amounts and this opens the door to many other potential bacterial agents and disguises itself as a typical strep infection in the earliest stages. It also suppresses T cell activity, and enhances susceptibility to endotoxin shock. As a group, the strep apparently cause an overall suppression of the immune system making the body hypersensitive to the effects of the toxins. It is also known that antistreptococcal antibodies sometimes attack the hosts own tissues and leads to autoimmune damage of the heart. The enzymes of group A and B are serologically different even though they may be the same type of toxin. By using both groups, weapons may be made hyper effective as any host immunity will likely not cover both types.

The bacteria and toxins should be incorporated into ordnance as enhancements enabling other more lethal organisms and toxins to invade and cause disease. If the strep infection alone is used and is extensive enough, it can cause meningitis resulting in death or brain damage making it an effective natural appearing weapon.

An additional note on toxin concentration and preparation for use in weapons will be added here. In examining the literature, I have encountered the use of freeze drying to remove water from concentrates of cell filtrates a number of times. By adding the dry protein concentrates to the dust or aerosol solvents in the ordnance, a safe and effective way of drying and incorporating the toxins into the weapons is achieved. If freeze drying equipment is available, it can be used to produce effective and stable bacterial and toxin based weapons of high efficacy. Using direct or added heat to evaporate water from proteins usually damages them and makes them useless as weapons. Air drying or vacuum evaporation at air temperatures has also been used with good results in toxin viability.

One final note - The author has experimented with the use of commercial blood meal used in livestock feeds. It produces a partial hemolysis when added to gelatin and for growing organisms that normally produce complete hemolysis. This is likely due to the breakdown of the blood components into some unusable substrates during cooking. It does not substitute for serum in streptolysin production but can be used as a poor mans culture plate substitute for identifying hemolysis.

Mycobacterium
(Tuberculosis and Leprosy)

<u>History and Recovery from Nature</u>

Tuberculosis is an ancient disease of man. The first evidence of tuberculosis has been observed in the bones of Egyptian mummies. The first isolation of the causative organism was achieved by Koch in 1882 who isolated and grew it in pure culture. He also reproduced the disease by inoculation with the bacilli and then recovered the bacilli in the host rabbits and guinea pigs which led to the development of Kochs postulates for identifying and associating disease with the causative organism.

It has been the largest cause of death (morbidity and mortality) in human history, exceeding that of malaria, and was the largest cause of death in the United States until early in the 20th century. Urban death rates exceeded 440 per 100,000 people in the early nineteenth century in the US, but has been reduced to only 2 per 100,000 by 1975. By 1980 there were only 27,000 cases with 1,770 deaths which still makes it the leading cause of death among communicable diseases. The WHO (World Health Organization) estimates that there are 20 million cases of TB annually worldwide with more than 2 million deaths due to M. tuberculosis.

Indians are affected at 10 times the above rates while Eskimo's are 30 times more susceptible. Affected individuals have historically been quarantined in sanitoria but recent advances in treatment have reduced the length of time required for treatment drastically. Human TB is passed from man to man while bovine TB is passed from cows to humans, usually from contaminated milk.

M. tuberculosis required X rays, clinical symptoms, and the recovery of acid-fast bacilli in the sputum to diagnose. Growth in culture required 20-22 hours for each generation while contaminating bacteria would often divide in 20-30 minutes and these would produce end products that inhibit mycobacteria making it nearly impossible to isolate. This required development of media and techniques to suppress other bacteria allowing it to finally be grown in isolation.

This organism is an intracellular parasite that may be seen in the macrophages of the bone marrow, lymph nodes, and liver of hosts with disseminated infections. Tissue biopsy's provide the easiest means of obtaining uncontaminated samples. Draining sinuses will have mixtures of bacteria which make isolation difficult.

All mycobacteria grow in at least small amounts on ordinary media except for M. leprae which causes leprosy. Long chain fatty acids in the routine media act to inhibit normal growth of mycobacteria. This inhibitory effect is nullified by adding egg yolk, albumin, charcoal or serum to the growth medium allowing it to grow slowly.

For best growth, the media used are solid, inspissated serum, egg or potato. In the 19th century it was almost impossible to get the mycobacteria to grow on culture media. It was finally found that a medium of whole eggs, potato flour, glycerol and salts could be solidified by heating at 85-90 C for 30-45 minutes (inspissation) would support the growth of these bacteria.

The generation time of mycobacteria is much greater than most other bacteria with one hour for the fastest growers to 20-30 days for a single division of M. leprae. Most of the pathogenic strains take 3-4 weeks of growth before they become visible to the naked eye.

Mycobacteria grow at pH of 6-7.8 with the virulence best maintained at 6.8. At a pH of 6.0, the virulence is attenuated in subcultures. The optimal temperatures for growth correspond with the host species. Those that infect birds grow best at 40 C, humans at 37 C, and reptiles or fish at 25 C. Mycobacteria are strict aerobes and the pathogenic species require added CO_2 in the air or medium for optimal growth and the recovery medium should contain added CO_2 as a gas and as added baking soda.

The most frequent means of obtaining tuberculosis is from droplets in the air from infected individuals which are inhaled. The second most frequent mode of infection in nature is ingestion of foods such as unpasteurized milk from tuberculosis cows. M. bovis is endemic in undeveloped countries and can easily be recovered from untreated infected milk and meat samples. The incidence is believed to be 70-100% in herds with the animals kept in stalls and those with advancing age having the highest rates (as in human populations).

Specimens are obtained from infected individuals from sputum, laryngeal swabs, affected tissues by biopsy such as liver, bone marrow and lymph nodes, urine, cerebrospinal fluid, and gastric lavage. Early morning urine samples are the most common used by the laboratories for recovery and culture. Solid pieces of caseous material should be extracted from the sputum for preparing slides or cultures. This is because mycobacteria are often encased by the mucoid or caseous material and must be dislodged and concentrated before inoculation or observation.

Many specimens are often needed to culture and isolate the organism. The high cell wall lipid content protect the mycobacteria from strong acid (5% oxalic acid) and alkali (3-5% sodium hydroxide) solutions which kill most other bacteria. For this reason, samples collected are treated with a decontaminating agent of acid, alkali, phenol or other material to kill off competing microbes and liquefy mucous liberating the cells. After a period of time, the agent is neutralized and the mix is usually centrifuged to concentrate the bacteria for inoculation.

Other decontaminating agents include trisodium phosphate (13%), benzalkonium chloride (Zephiran), and both. These are used overnight and treated specimens are then inoculated into egg base media which neutralizes the growth inhibiting effect of these substances. Lecithin can also be added to agar cultures to neutralize Zephiran.

Mycobacteria can also be selectively recovered using a concentrated solution of 2% sodium hydroxide with n-acetyl-l-cysteine (NALC) which liquefies mucous by splitting disulfide bonds. This action liberates mycobacteria and these can then be readily sedimented for inoculation by centrifuging. The sodium hydroxide is increased to 3% in hot weather or with samples containing heavy levels of contamination. A mix called "Sputolysin" is commercially available for this type of decontamination of samples.

Other homemade decontamination mixes can be prepared using 20% sulfuric acid with 20 g of ferrous sulfate per 100 ml. Hydrogen peroxide at 3% can also be used and these are added to the specimen for up to one hour with shaking and mixing.

M. tuberculosis is unable to convert free niacin to niacin ribonucleotide. This causes water soluble niacin to accumulate in an egg base culture medium which helps in identifying this species. Reagent impregnated filter paper strips which turn yellow in the presence of the niacin are used to identify it. Another way of identifying it is to add cyanogen bromide in the presence of aniline to the colony which causes it to turn yellow.

Leprosy is an ancient disease of man. It was accurately described in 600 BC in India and was recorded at the time of the pharaohs in Egypt. It is now most common in Africa and Asia and is prevalent in South America with endemic centers in Brazil, Columbia and Argentina. There are around 12 million cases known worldwide. M. leprae is the causative organism and is a true parasite of humans and is difficult to culture. It has been called "Hansens disease" to deter the social outcast effects of the disease. It is most common in central Africa and is also found in North America with leprosaria in Tracadie, New Brunswick and Carville, Louisiana. Intimate contact is required for infection and the incubation period is 2-4 years. The disease is chronic and the mortality rate is high.

In endemic areas of Africa, about 20-50 people per thousand are infected. The primary infection route is through the skin via personal contact. In WW2, two US marines were tattooed in 1943 in Melbourne, Australia and both developed leprosy in 1946 in the tattooed areas. The leprosy cells are often recovered from the skin of infected individuals long before the lesions become visible making them highly contagious.

It was first observed under the microscope in 1872 with the round epitheloid cells being called lepra cells. Two types of infection are produced by M. leprae, the lepremetous type characterized by gross deformation with large numbers of organisms detectable in the lesions, and the tuberculoid type, also known as macula anesthesia. In this type, less dramatic lesions occur which contain fewer organisms and are insensitive to heat and touch.

Recovery of the organism is by obtaining aspirates from lesions and from biopsy materials. This is extremely dangerous as the infective capability is high. Most lab workers boil the samples before examination.

Growth and Production

Mycobacterium have a high lipid coat content in their cell walls and as a result they stain poorly with ordinary stains. A mordant such as phenol is usually used and heat is often applied during the staining to help it penetrate the cell wall. One of the best methods involves using basic fuchsin with phenol (carbol fuchsin) over heat. Once stained with heat, the bacteria retain the stain even when flooded with mineral acids, alcohols or other decolorizers. The ability to retain the stain has led to the term "acid-fast bacilli" which is used as a collective term for these organisms. The bacilli will also stain at room temperature with this method in about 18 hours.

The cell wall contains three layers surrounding a three layered plasma membrane. This makes it stain and survive unlike any other gram positive or negative bacilli.

Under the microscope, Mycobacteria are usually slightly curved with rounded ends or straight. They appear in groups of three or four or singly as slender rods measuring 1-4 *u*m x .3-.6 *u*m although some may be 8 *u*m long. Older cultures usually display the shorter forms. Human strains usually appear as long, thin, curved forms with granular staining.

They are non-motile, non-sporing, aerobic, and very slow growing. In tissue the cells may occur singly, in pairs often forming an obtuse angle, or in small bundles of parallel bacilli. They is very resistant to drying giving it good storage ability in ordnance. In old cultures, individual cells may grow into long filaments and show branching. The bacilli are sometimes seen in compact masses in which the individual cells cannot be distinguished.

In culture, long filamentous forms are sometimes seen with club shaped or swollen cells resembling the diphtheria bacilli. Individual cells often have a marked granular appearance that occur in abundance and appear similar to a chain of cocci.

Using gentian violet in alcohol with aniline oil instead of crystal violet causes the bacilli to stain gram positive. Fluorochrome dyes (auramine) are also used with acid-fast bacilli which appear as luminescent objects against a dark background when used with a properly equipped microscope. These dyes accept the high energy of ultraviolet light and then emit the light in a longer wavelength in the visible spectrum that we can see as luminescence. These organisms appear as bright yellow cells against a dark background.

Virulent strains of human tuberculosis often form characteristic microscopic "cords" in liquid media. The bacilli form in parallel arrangements in a long strand side by side and end to end to create the cords. Cord formation has been associated with increased virulence and when the cords are treated with petroleum ether, the filament breaks up and a lipid substance can be extracted which is highly toxic to mice and leucocytes.

One other extraordinary form of TB occurs in specimens of pus, lymph nodes or serous exudates in which no acid fast organisms are found. When these non-acid fast granules were first injected into other animals they also produced TB but would not grow on artificial media. This form of TB is called the "L" form and can now be induced in tissue culture by adding lysozyme and acid phosphatase. The L structures have no cell wall, are spherical with a granular structure and will revert to the acid-fast bacilli under suitable conditions.

All mycobacteria except M. leprae grow slowly on ordinary media. The media most often used in laboratory cultures is Loewenstein-Jensen medium which is a mix of coagulated whole eggs, defined salts, 5-6% glycerol as the preferred source of carbon, potato flour and .025g per 100 ml of malachite green. Adding glycerol and Tween 80 will often shorten the generation time and the strains that show enhanced growth in the presence of these substances are called "eugonic". Those that are slowed by its growth are called dysgonic. Adding aniline dyes such as malachite green or crystal violet in the inspissated medium helps to control unwanted bacteria.

Coagulated fresh egg yolks, potato flour, and glycerol alone (ATS medium) will support mycobacteria growth. Malachite green is usually added as an inhibitor. Adding 5-10% CO_2 will increase growth and produce larger colonies.

Penicillin, naladixic acid, cyclohexamide, carbenicillin, and lincomycin are also sometimes added to the culture to inhibit unwanted bacteria.

Colonies of mycobacteria appear tough and tenacious, dry and wrinkled with irregular edges (mamillated). M. tuberculosis looks like dry cake crumbs scattered over the surface of the medium. Most strains are very difficult to emulsify. The colonies are white and turn to a buff color as they age. If a fragment of the colony is inoculated on the surface of glycerol broth, the growth spreads across the surface as a grey veil-like film and gradually grows over the entire surface as a buff colored or white, wrinkled pellicle that thickens into a wrinkled membrane. If no surfactants are used, the colonies tend to spread up the sides of the flask. Some may become detached and sink to the bottom as a lumpy sediment. On solid media, the colonies will often have a nodular, heaped up appearance. Broth cultures need to be agitated to periodically add oxygen to the liquid for the most rapid growth.

M. mycobacterium will also grow on ordinary media or broth when 5% glycerol is added although growth is slow.

Most cultures of M. tuberculosis produce a pleasant fruity odor but plates should only be sniffed through bacterial air filters or the technician will likely become infected. They produce opaque colonies with all mycobacteria producing varying degrees of pigmentation. These range from cream to shades of lemon to orange to many shades of red. These pigments are used to differentiate species. Some pigments only develop in the presence of light which also aids in identification.

M. tuberculosis produces no pigment except for a light buff color when exposed to bright light. The procedure is to expose a grown colony to bright light such as a 100 watt tungsten bulb or fluorescent equivalent for 24-48 hours. A pigment develops and is compared to a similar culture control that is kept in the dark. Some species produce pigment only in the dark while others do so in the light. M. tuberculosis is considered to be non-pigmented or lightly pigmented.

M. tuberculosis grows well at 37 C but not at all at 30 C or 42 C. Colonies are usually detected at 12-21 days but some strains take up to 6 weeks for visual growth to be seen so growing this organism requires a great deal of patience. Production for weapons use is usually accomplished by preparing a broth based on the solid media described with added CO2 and oxygen. A surfactant called Tween 80 is added which supplies water soluble lipids and allows for diffuse growth by acting as a dispersing agent. Vigorous shaking will also disperse the organisms. This medium becomes turbid in several days indicating large scale production and growth. Broth can also be prepared using casein hydrolysate, bovine serum albumin, asparagine, salts and Tween 80. It can also be solidified using agar.

Saprophytic species produce colonies that are distinguished from those above by appearing as shiny, butyrous colonies with an even periphery.

Inoculated agar is grown for up to six weeks at 37 C for recovery of the organisms in culture. The media is examined at 48 hours, at five days, and then weekly. When significant growth appears, they should be examined to make sure that mixed colonies are subcultured and separated.

None of the usual carbohydrates are fermented and most strains produce varying amounts of catalase. Human M. tuberculosis produce excessive amounts of niacin which is measured and tested for in identification.

M. leprae is more acid fast than other mycobacteria and 1-40% sulfuric acid is used as decolorizer in some staining methods. The bacilli appear similarly to M. tuberculosis and arrange intracellularly in large groups or pockets resembling packets of cigars and this arrangement is retained even if the cells are ruptured. It is a straight or slightly curved bacillus about the same size as the tuberculosis bacilli having club shaped or pointy ends. It is gram positive with normal stains and is non-motile and non-sporing.

M. leprae are long slender rods that are non-motile and do not produce spores. They produce capsules that are destroyed by hot carbol-fuchsin staining.

Toxicity and Harm

Smooth and rough cultures are encountered on the same medium and most smooth cultures are avirulent while most rough cultures are virulent. Strains obtained from culture collections are classified as -

H = Human variety
Number = designates the strain
R or S = Rough or smooth
A or V = Avirulent or virulent

The strains are recorded as [H37RV] designating it as a human bacilli, strain 37, rough colonies and virulent. H37SA would be the same strain that is smooth and avirulent.

The organisms enter the body by inhalation or ingestion and occasionally through the skin making it useful as a weapon by all three routes. Most of the organisms are phagocytosed rapidly and do not become infective.

In typical infections, the lymph nodes of the trachea and bronchi and the lungs are colonized producing an acute inflammation. Within a few weeks the organism -

1. Produces a caseous necrosis in which the infected tissue cells die off en masse producing the microscopic tubercles characteristic of the infection.

2. The organisms lyse limiting the infection to a minor scar.

3. The organism spreads to neighboring tissues and other body sites producing a variety of infections such as nephritis, meningitis, and osteomyelitis.

The tubercle bacilli "seed" a tissue after which the body produces an immune response. The body's efforts to fight off the infection results in the formation of the granulomas which are called tubercles. These usually act to contain the spread of the cells. These tubercles are visible to the naked eye and give the disease its name. When a blood vessel is near and experiences erosion during this process, large numbers of the bacilli make it into the bloodstream and spread throughout the body, usually resulting in high fatality rates (military TB).

The organisms may enter the blood but have never been cultured from blood samples. When they enter the blood in large numbers it has been called "military" tuberculosis and is almost always fatal if untreated.

[Authors note: In WW1, the Germans inoculated an entire village with a "Vaccine" which was actually tuberculosis. It is believed to have become an avirulent strain from subculture by the time of injection and almost no one became infected.]

At early stages of infection, the host develops a hypersensitivity to the "tuberculo-protein" which can then be detected in the tuberculin test.

The tuberculin test is accomplished by taking a preparation of tuberculo-protein and inoculating it intracutaneously. A delayed type of hypersensitivity can be detected as varying degrees of induration to the protein. A positive test means that the individual has been exposed to TB in the recent past or may still harbor the organism. False negative tests occasionally occur but usually the negative results mean that the person is free from infection.

The incidence of tuberculosis increases as living conditions decrease as is seen in urban ghettos. Those exposed to inorganic dusts, especially silica based dust have high rates of the disease which commends the use of silica dusts in weapons as a carrier of the organism.

When the tubercle bacilli are inoculated into tissue cultures, they are internalized into the cells and lie in cytoplasmic vacuoles within the host cells. Once inside these vacuoles, the virulent strains grow more rapidly than avirulent with the growth in 3-5 days destroying the host cells. The virulent strains also inhibit the fusion of lysosomes in macrophages which allows their easy determination of virulence.

The guinea pig is very susceptible to infection by subcutaneous injection. In a few days a local swelling results with tubercle nodules which become confluent, undergo caseation and finally ulcerate. The nearby lymph glands become infected as the bacilli spread along lymphatic channels throughout the body. The animal loses weight and dies in six weeks to three months. At autopsy, the lymph glands have the characteristic lesions, the spleen is enlarged with grey-white nodules or larger caseous lesions and the liver is similar in appearance. The lungs and kidneys may be free of lesions.

Less virulent strains are more sensitive to hydrogen peroxide (Mitchison 1960,63). Mice are less susceptible to tuberculosis than the guinea pig which can be infected with only a few cells, but after intraperitoneal or intravenous injection they also develop progressive or chronic lesions which depend on the dose and the virulence of the strain. The human strains have been found in monkeys, cattle, pigs, and dogs.

The bovine tuberculosis has less growth than the human type. Glycerol does not improve its growth rate and it is more virulent to cattle. It is fatal in the ox while the human form only causes a local lesion which heals spontaneously. The two types can be distinguished in rabbits. Injecting intravenously with a saline emulsion of .01-.1 mg of colony from solid medium, the bovine type produces a generalized tuberculosis with death in 3-6 weeks. The rabbits survive the human type or may die in several months with lesions confined to the lungs and kidneys. Subcutaneous injection of 10 ml produces fatal tuberculosis with the bovine type while the human type produces a local lesion only.

Avirulent strains may be made virulent by a pass through test animals (or multiple passes).

Mycobacteria have a unique method of transporting iron. They produce a lipid-soluble iron chelator called mycobactin that is present in the cell envelope and has a high affinity for iron. It also serves as a mediator of iron transport and internalization. Because it permits mycobacterium to compete more effectively for available iron in host tissues, it has been suggested that it acts as a virulence factor.

M. tuberculosis also produces an extracellular, water soluble substance called exochelin which effectively robs iron from the short term storage in mammalian cells (ferritin). The iron is liberated and solubulized by the exochelin and then when brought into contact with the cell wall, it is taken up by the mycobactin and transported through the cell wall. This process found only in the mycobacteria genus is unique among all bacteria.

This organism does not produce any known toxin and no structure or mechanism has yet been described as being able to explain how it becomes virulent in the host. The only directly associated condition with virulence is the formation of cords which look like paired string. non virulent strains do not produce this effect. A substance called cord factor is associated with this growth characteristic. This cord factor also attacks mitochondrial membranes causing functional damage.

Leprosy produces two types of infection. The nodular (lepromatous) has nodules forming in the skin, mucous membranes, and various organs. The tuberculoid type has the granular tissue spreading to nerves which leads to sensor and motor paralysis resulting in anesthetized skin areas. Both types can occur in the same host. The organism is found in the lesions in large numbers especially in the nodules. They are distributed intracellularly with parallel bacilli occurring in bundles which may completely fill up the cells. Mucosal cells ulcerate readily and cause the secretion of bacilli in the mucous from the nose and mouth. These organisms may be used directly as weapons and are highly infective.

In tuberculoid leprosy, cellular immunity holds the infection in check and the effects are usually localized affecting only the skin and nerves. It progresses slowly, lasting about 18 years. In lepromatous leprosy there is little or no immune response with the disease becoming acute and characterized by the development of masses of granulation tissue called leproma. The coalescence and growth of these lesions produces the distortion and mutilation seen in the leper host.

The organism is found in enormous numbers in this last type in the lesions and are recovered in large numbers in nasal secretions (up to 100 million cells daily). They survive drying and remain viable for 1-2 days. Very few are found outside the tissue cells. They are found in the cytoplasm but do not invade the nucleus. They are capable of invading every type of cell and tissue with the most common sites being the skin and nerves.

Until the early 1960's, it had been impossible to produce animal model infections for leprosy. In 1961, Shepard found that the bacilli would grow when injected in the footpads of mice. This was followed by use of radiation and thymectomy to produce immunosuppressed rats and mice which could be infected successfully. The organism can also be grown in the ears and footpads of hamsters and is believed to be due to the lower than body temperature in these sites.

In the early 1970's Armadillos were observed to develop nodular lesions in which the bacilli would grow to enormous numbers. The animal turned out to be a good host because of its lower body temperature and weak cellular immunity. It was also found that the species is naturally infected with leprosy. The animal constitutes a good source for large numbers of infective bacilli that can be used as a deadly covert weapon whose effects are not seen for years. This organism is highly infectious and demands that strict isolation be instituted to control its spread.

It is known that, like TB, leprosy has a high degree of transmission and low attack rate in endemic areas that is due to the cell mediated response (and possibly due to small differences of produced body heat, temperatures below 30 C where the organism can easily initiate infection- the nose, ears, etc). It is known that man has a high degree of immunity to M. leprae and it probably requires a high dose to be successfully infective. A number of attempts have been made to experimentally infect humans but most have ended in failure.

<u>Protective Measures</u>

Mycobacteria are more resistant to chemicals and drying than other vegetative bacteria and this is due to the high lipid content of their cell walls. This high resistance coupled with their high pathogenicity means that they are very dangerous to handle in the laboratory. It also makes them very effective and hard to eliminate weapons. They resist drying and many cells survive for weeks or months if kept out of sunlight. They can also resist phenol at 5% for several hours. They are very susceptible to sunlight and ultraviolet light, even through glass.

In preparing organisms in culture you should have -

1. An inoculating hood equipped with a UV light and a good exhaust system.
2. Masks, gowns and gloves should be worn only once and incinerated.
3. Pipetting should never be attempted.
4. Shielded bunsen burners should be used to prevent contamination from spattering.
5. Work areas should be irradiated with UV light for at least 12 hours after each use and washed down with 5% phenol.
6. Cultures should be disposed of by autoclaving at 126 C for 20 minutes.

Historically, streptomycin, isonicotinic acid, hydrazide (INH), paraaminosalicylic acid (PAS), ethambutol, rifampin, viomycin and some other antibiotics have been effective to varying degrees. Combinations of drugs are usually used over long periods to treat infections. Recent strains have become very drug resistant and are the best candidates for weapons. They can also be made drug resistant by mutation and subculture as described elsewhere. Modern drugs based on the "sulfones" and the pyrimidine carboxylic acid derivatives are used today with resistant strains. The sulfones are highly toxic and are rarely used.

A bovine strain has historically been used as the vaccine which is given to those who test tuberculin negative. The immunity that is produced is relative to the infective dose rather than absolute. Killed vaccines usually gave no protection at all. In 1921, a live vaccine prepared from a bovine strain subcultured for 13 years on bile-potato medium was given orally, and then became popular by intradermal injection. The protection given by the vaccine has been estimated at 80% for up to 10 years. It accomplishes this by causing a successful infection without producing the disease by a means that has not been determined yet. It is known that it does not prevent all infections but will inhibit the generalized spread of the disease. It also still causes disease in hosts that are immunodeficient.

Fresh air, good food, and pleasant surroundings contribute significantly to rapid convalescence of patients. Overcrowding, dusty environments that are poorly lit are the worst conditions for tuberculosis spread between individuals and fomites or dust saturated air.

M. leprae is treated with prolonged therapy using diaminodiphenyl sulfone which may suppress the disease to the point of convalescence. Complete cures are very rare.

<u>Incorporation into Weapons</u>

Man is susceptible to mild infections of M. tuberculosis and this is demonstrated by an almost 90% rate of positive tuberculin tests in many populations. He is much more resistant to the disease of tuberculosis and this may also account for the failure of the German inoculation operation in WW1.

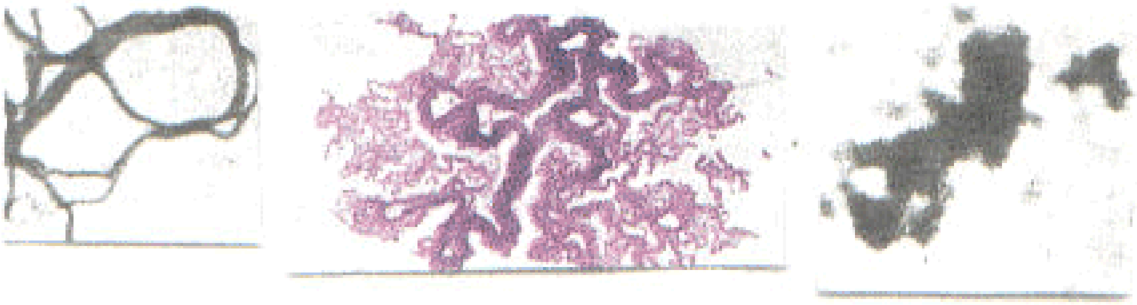
The use of silica or fiberglass a carrier for dust based weapons is highly recommended. The higher the concentration of dust and organisms, the higher the infective rates will be. Even if the mortality rate is not high, the fact that it is contagious can easily enable the widespread epidemic effects of an attack and quickly overwhelm the ability of an enemy to contain it through quarantine.

Bacilli that are dried into dust particles below 5 microns in size and stored in the dark at 37 C are capable of initiating infection for up to 8-10 days and in dried cultures for up to 12 years. They are resistant to heat and most chemicals such as hypochlorites and disinfectants in this state. This allows for their use in treated water supplies, food processing and service targets and other public institutions with good sanitary practices. It is infective by <u>all</u> routes of exposure including eye, dermal via cuts or abrasions, ingestion, inhalation and genitourinary. In order to produce potentially fatal disease by inhalation, the micron size must be below 5 in order for the bacilli to enter the alveoli and adhere to the mucosal surfaces there.

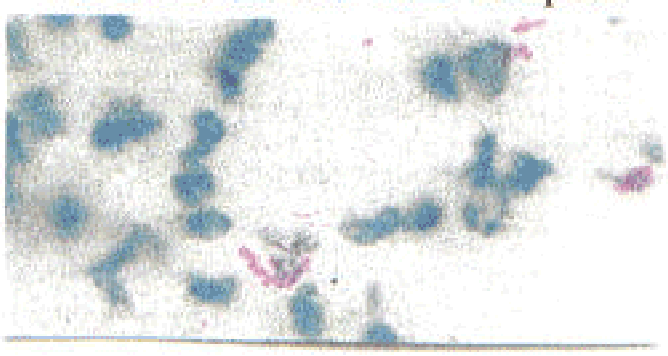
In guinea pig tests it has been found that lethal infection can be created against vaccination with doses about 1,000 times that necessary against unvaccinated animals. It is unknown whether this will apply to human targets although the author thinks it is likely. It has also been found that a VA hospital patient with tubercular laryngitis was able to contaminate the air in his room so thoroughly that when the air was pumped to an adjacent room with 15 guinea pigs, all of them became infected.

Experimental animal tests indicate that infection via the eyes is easily accomplished although no human tests have been conducted. Mortality rates are almost always determined by the invasion of the blood stream by large numbers of virulent cells. TB can invade virtually every organ once it gains access to the blood stream. The ability to successfully infect depends on the body's immune response and this commends the use of other stress inducing biologicals in combination with TB which mirrors the high mortality rates experienced in the natural occurrence of the disease.

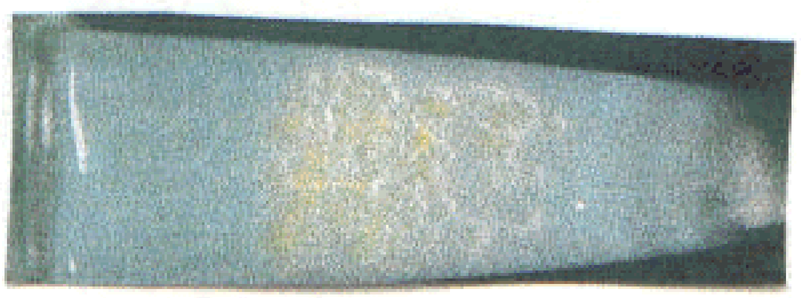
Cord growth in a virulent strain and an avirulent culture.



Tuberculosis from urine samples.



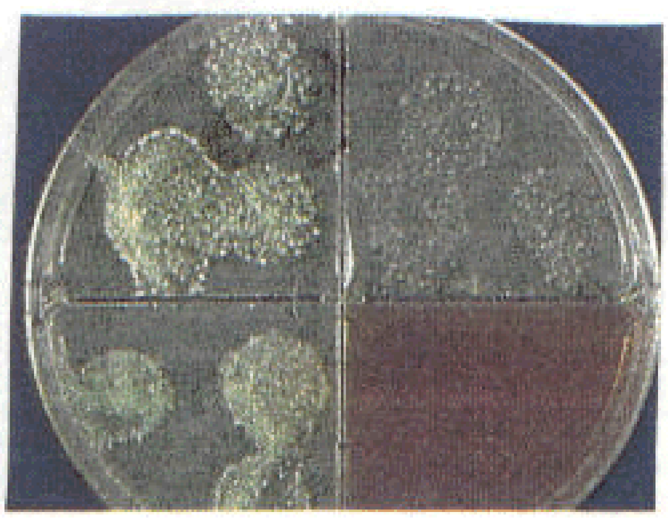
Buff colored, raised center, rough and flat colonies of M. tuberculosis on Lowenstein-Jensen media.



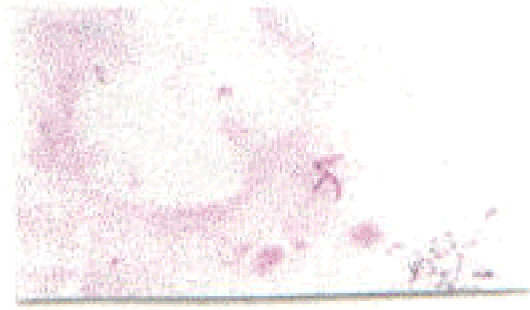
Thin, spreading colonies on agar.



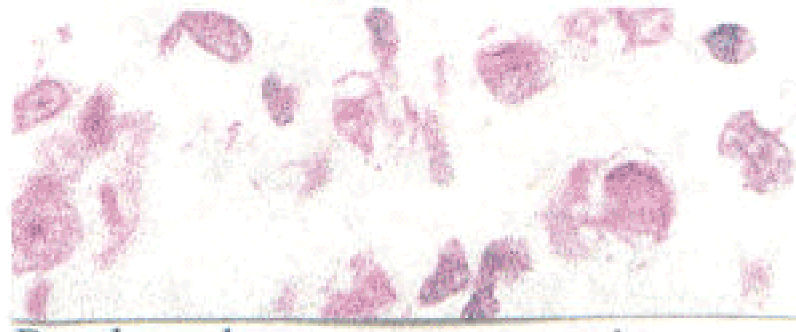
Sensitivity test of tuberculosis grown from 10 day broth cultures.



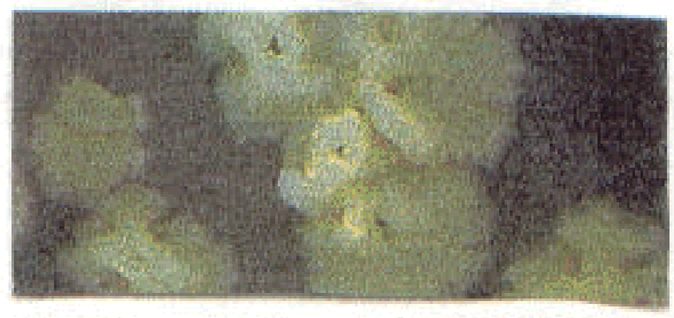
A small group of M. tuberculosis at obtuse angles near the center.



Bundles of red stained leprosy bacilli inside of host cells.



Bread crumb appearance seen under dissecting scope of M. tuberculosis colonies.



M. tuberculosis on left, and M. kansai grown in light (middle) and dark (right).



Small lesions with ill defined edges from rabbit injected with .04 g. bacilli [Lungs, 5 weeks after infection]



Francisella Tularensis
(Tularemia)

<u>History and Recovery from Nature</u>

Francisella tularensis, the bacteria that causes Tularemia has previously been classified as a member of the Pasteurella genus. It has been reclassified and named after Edward Francis, the American bacteriologist who first studied the organism. It had been identified by some workers with the Brucella family because of its unusual growth requirements and serological relationships with other members of that group. It was first discovered by McCoy and Chapin in 1912 as a plague-like disease of the California ground squirrel. It is usually acquired by man from rabbits via the bite of infected deer flies.

F. tularensis is a strict aerobe and does not grow on ordinary media. Enrichments such as cysteine, animal serum, or egg yolk must be added to enable it to grow. It also does not produce catalase and this combination helps identify and differentiate it from other members of the Pasteurella genus.

It grows between 24-39 C with 37 C the optimum. It will grow slowly on pure egg yolk, and rabbit spleen is sometimes added as a homemade recipe to blood agar to culture it (rubbing the spleen across the surface of the blood agar provides the essential growth needs). Horse serum with .1% cysteine and 1% dextrose added is also used. Increasing CO_2 to 5% aids in its isolation. It will also grow on chocolate-cystine agar.

The organism forms acid but not gas from glucose, maltose, and mannose. When it is grown on a media containing cysteine, it tests positive for H_2S with lead acetate paper. The best way to inoculate the medium is to aspirate the suppurative material from a lesion. or bubo (or animal tissues) that has been formed with a syringe or small pipette. At least 5 ml of inoculum is placed in the center of a cysteine, glucose, blood agar and is spread evenly across the entire surface with a sterile glass rod.

Tularemia is endemic in many small wild animals, especially ground squirrels and is believed to be in the tissues of at least 1% of the US wild rabbit population. It is usually transmitted to humans from the bite of a blood sucking arthropod. Direct contact, bites, and scratches are the most common means of infection to furriers, trappers, and animal handlers who account for the vast majority of mortality and morbidity from tularemia. These workers often acquire the disease by handling the infected flesh of these animals. It is firmly established among rodents in the western US and is easily recovered in this area of the world although it can be found almost anywhere on earth. Carnivorous animals may also acquire the disease from eating the infected flesh of other animals and in this way helps maintain a reservoir for further infection.

It can be cultivated from ticks (especially wood ticks) and other arthropods as well and is even found in bird populations and outbreaks may be seen in a wide variety of animals such as muskrats, beavers and 48 other types of vertebrates. Stream related outbreaks have been traced to infected beavers. Waterborne epidemics have also been reported in Asia and Europe. Infection of natural waters is common in the northwestern US. The organism may persist for months in water and mud and evidence suggest they may even multiply there. This offers another means of recovery from nature. In the case of the wood ticks, the organism is passed on from the adult tick to the egg and both the larvae and the nymphs are infectious. [This allows the use of remotely delivered ordnance based on this type of vector].

Asymptomatic infections are known to have occurred as outbreaks due to positive skin and serological tests of persons living in endemic areas and reporting no previous clinical infection.

In the western US, a sheep epidemic may occur with mortality reaching 10% and subsequently spread to those individuals handling the animals and their wool. Monkeys have been tested experimentally with the organism for biological weapons use and the LD 50 ranged from a low of only 14 cells with the smallest aerosol particle sizes and increases with increased micron sizes of the particles. The average incubation period is 1-6 days followed by the acute illness lasting up to 11 days. In lower doses, most of the animal survive and are negative within two months. Autopsy shows significant lesions in the lower respiratory tract with necrosis, pleural effusion, and adhesion involving the lymph nodes.

It is easy to identify F. tularensis from fluorescent antibody staining using exudates and tissues containing the organism. Cultural isolation usually requires persistence and patience for successful recovery and production of this organism. It can also be identified with agglutination in specific antiserum.

The infective dose for man is very small based on the monkey tests and the incidence of human infections from handling tissues and cultures with full body protection.

F. tularensis is capable of penetrating unbroken skin through the tiniest break or directly through the pores and this has caused many accidental infections in the lab. This ability also makes it an excellent candidate as a military weapon. It easily invades through the eyes, mucus membranes and abraded skin. It has also been spread from the excreta of water rats into water supplies where it infects those drinking the water with a high mortality rate.

A small pustule forms at the site of inoculation and the organism invades the lymphatics producing a necrotizing inflammation and enlargement of lymph glands. These lesions are called buboes and are similar to those seen in bubonic plague. The form of the disease that is inhaled in dust or ingested with food in nature is very rare but yields fatality rates over 90% and is comparable to plague in its ability to kill when used in this form. Those who survive experience a long, febrile illness.

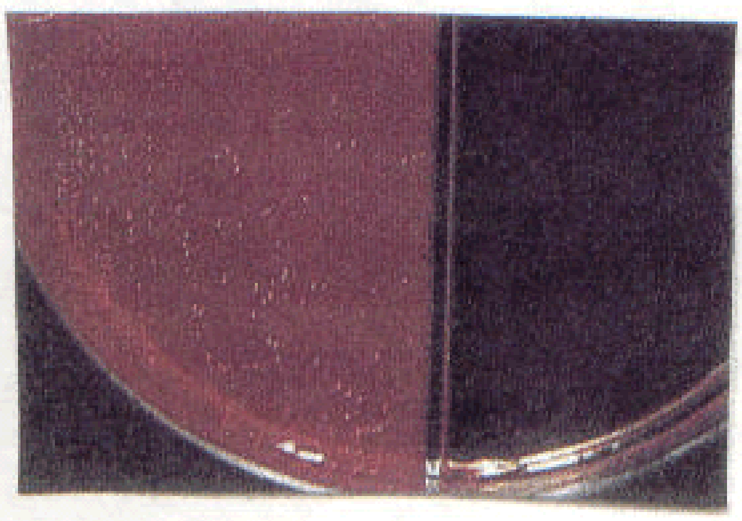
F. tularensis stores for many years when frozen but does not survive well in cultures stored at room temperature or refrigerated.

Growth and Production

F. tularensis is a tiny gram-negative, pleomorphic cocco-bacillus or rod that yields a bipolar stain. It measures .2-1 *u*m x .3-.7 *u*m and bean shapes, L shapes, dumbbell shapes, spermlike shapes and many other forms have been observed. These bizarre forms give the impression that the organism reproduces by budding rather than binary fission. It stains best with dilute carbol-fuchsin and when it is recovered from the spleen or liver of infected animals its capsule is evident. It multiplies as an intracellular parasite as evidenced by its growth in these tissues. Smears from the spleen of infected mice or guinea pigs appear as coccoid forms in well defined clusters.

When cysteine and dextrose is added to blood agar, 1-2 mm dia., clear, droplike colonies are finally visible in 48-72 hours after inoculation. A slight greening of the agar is seen just underneath the colonies, usually after prolonged growth of 1-2 weeks. This also allows for a presumptive identification. Colonies are mucoid and easily emulsifiable. It does not form spores and the organism is non-motile. Plate cultures are usually positive in 1-5 days but can take as long as three weeks to produce visible growth.

A 48 hour growth of tiny, mucoid colonies on enriched blood agar and no growth on MacConkey.



High power view shows the tiny colonies streaked on the plate.



Toxicity and Harm

There are five clinical types of tularemia. Ocuglandular, ulseroglandular (skin), glandular (bubonic), typhoidal, and pulmonary. In the first two, the organisms will pass through the lymph nodes and develop into septicemia and occasionally meningitis. The ocuglandular form is the most common one acquired by lab workers.

The most common form of human infection and animal spread is by vectors such as fleas, flies, lice and ticks. The usual route is rodent to rodent and rodent to man. Test of the organism is made by inoculating guinea pigs or mice with exudate from glands or ulcers.

The bacillus produces endotoxin and a different heat-labile toxin. The more virulent strains of F. tularensis produce acid from glucose and possess a citrulline ureidase system. It is found most often in North America while the less virulent varieties are found worldwide.

The glandular form of the disease begins with headache, fever and pain. A papule appears, usually on a finger where the bacilli presumably entered the body. This later breaks down and forms an ulcer. Lymph glands become painful and swollen and may break down discharging the purulent mass.

In the eye infections, the inner surfaces of the eyelid and glands become tender and swollen. In the typhoidal infections, there are no local symptoms and a pleuropulmonary disease develops which may be confused with other similar disease.

The bacilli may be found in the blood during the first week but is rarely cultured from it. During this time the initial bacteremia may develop which develops into septicemia in fulminating cases. Tularemia is characterized by Intracellular parasitism in which the bacilli grows within or on various cells. It has actually been grown on tissue cultured alveolar macrophages which accounts for its extraordinary ability to survive during its initial invasion.

Agglutinins appear in the blood in the second week of the disease and may be present in diminished amounts for up to 18 years. With treatment, the disease lasts about 2-4 weeks and the fatality rate is low, about 5% for those cases where the organism is acquired through the skin and not inhaled or ingested.

Cultures of the organism can be injected into guinea pigs with death at 5-10 days and the characteristic pathology evident at autopsy.

Protective Measure

F. tularensis is killed by pasteurization at 56 C in 10 minutes. It is sensitive to streptomycin (which drastically reduced its mortality rates) and tetracycline's but resistant to most other antibiotics. Animal, especially "lazy rodents" must be handled with care to minimize contact with the organism and reduce the chances for infection. Surviving an infection seems to confer some immunity but vaccines have proven ineffective, especially against the inhalation and ingested forms.

The use of full body protection and safety hoods with discharging air flow and filters is recommended when handling large scale cultures of this organism.

Incorporation into Weapons

The cultured cells should be modified for resistance to Streptomycin for maximum effectiveness and this appears easy to accomplish. The mass culturing of the organism requires much time and care because of its slow growth rate and small volume. It should be handled in closed systems only because of its remarkable ability to infect virtually any human that comes in contact with only a few of its cells.

F. tularensis is capable of producing very high rates of infection by skin contact alone. When inhaled or ingested with food or water, it produces mortality rates rivaling that of plague. This makes it ideal for almost any type of weapons use including assassination. It can be transmitted by most insect vectors allowing for remote delivery. It is also effective as a large scale weapon like plaque except that after the initial outbreak, it does not easily spread in the same manner making it a safer to use area weapon.

The weapons should include small amounts of cysteine, dextrose, glucose and/or rabbit spleen to aid in its germination in human tissues.

Yersinia
(Plague, Black Death)

History and Recovery from Nature

Yersinia is named after the French bacteriologist Alexander Yersin who first identified the bacteria Y. pestis as the causative organism of the plague in 1894 (along with Kitisato who also discovered it a short time later independently). Many of the Yersinia group were originally classified as Pasteurella and have been reclassified into their own genus. The most potent weapon of the genus and probably all bacteria based weapons is the Y. pestis which is endemic in rodents in most of the world.

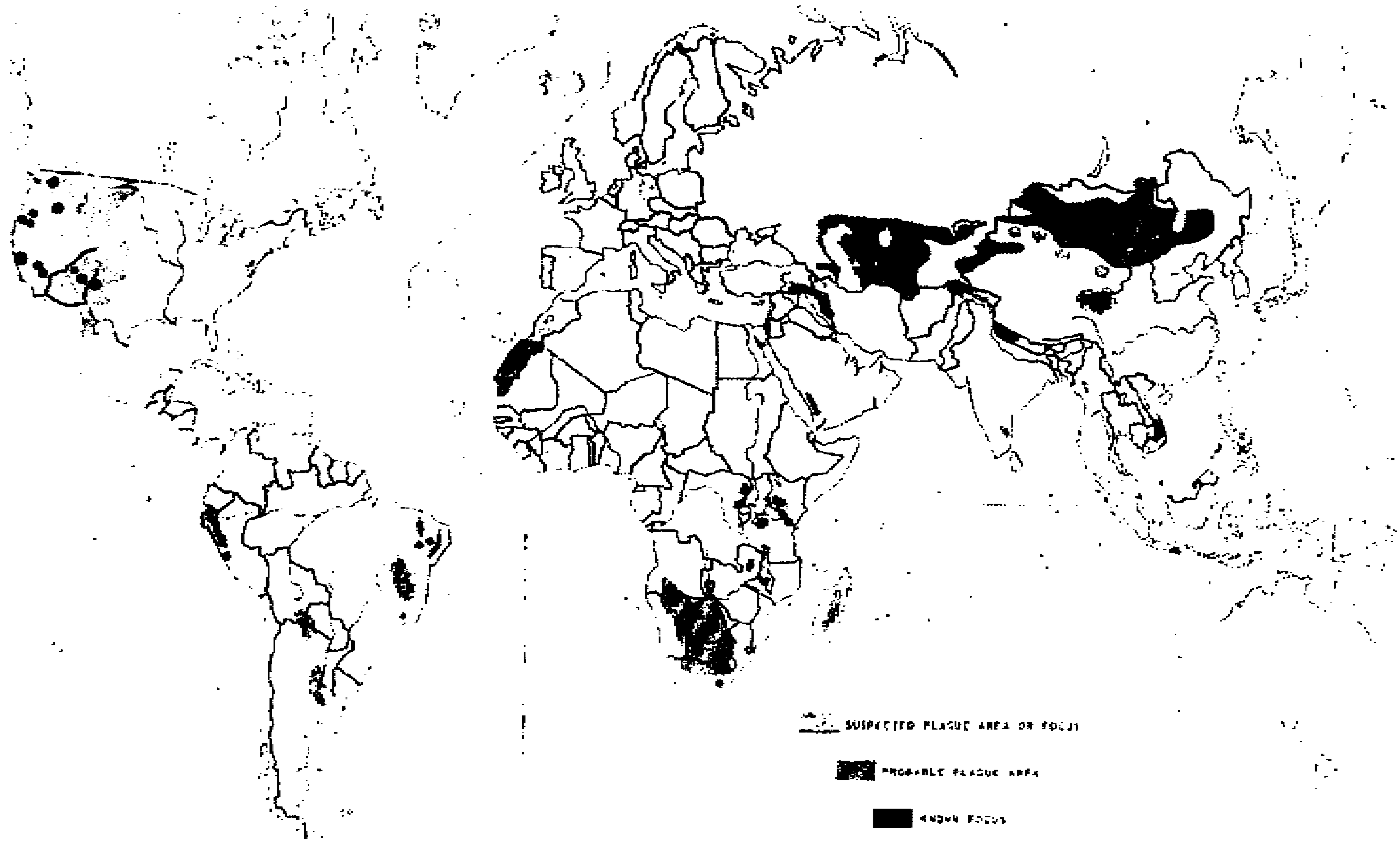
There are sporadic cases in the US, mostly in the southwestern United States, with 523 cases reported between 1900 and 1951. It is responsible for the "black death" that killed app. 25 million people in Europe and Asia in the 14th century. This was believed to be 1/4 of all living persons in this time period. Earlier, in a matter of a few decades, over 50% of all inhabitants of the Roman Empire died making it the quickest mass killer by bacteria in all of human history. It is believed to be the biblical plague of the Philistines in 1320 BC and the pandemic plague during the reign of Emperor Justinian in AD 542.

Many of the plague outbreaks in history have left an indelible mark on historical writings leaving behind detailed evidence of their occurrence. A realistic description of plague is provided in the "Journal of the plague year", a fictional account of the devastation of the plague outbreak of London in 1665 which killed more than 70,000 people. After periods of quiescence, plague again appeared in epidemics Hong Kong in 1893 and Bombay in 1896. From 1896 to 1918, more than 10 million people died from Y. pestis in India.

The first recorded case in the western hemisphere occurred in 1899 in Santos, Brazil and it finally reached the United States in 1900 in San Francisco. It is believed to have been brought ashore by infected rats aboard grain ships. The infection spread rapidly to ground squirrels and other wild rodents resulting in a major epidemic with 167 cases in 1907-1908 in San Francisco. Most of the cases involved hunters who came into contact with infected animals. It is now endemic in much of the US west of the Mississippi river. The area south of San Francisco is also well established with infected fleas and rodents. At least 57 species are known to be permanent foci of plague bacilli.

Plague is endemic in rats and other rodent populations and is spread by rat fleas. Almost all human infections originate from rats to fleas to man with the resulting bubonic form. The transmission begins with the flea sucking the blood of an infected rat and ingest the Y. pestis. The bacilli multiply in the stomach of the flea and eventually block its pathways with bacillary masses. When the insect bites again and sucks blood, it regurgitates sending part of the mass into the bite wound infecting the new host. The flea may also discharge the bacilli in their feces into the wound which also contaminates the site. The plague and its flea hosts live longer in cool temperatures such as 10 C (50 F) with temperatures above 80 C being unfavorable for outbreaks.

Known areas where plague is established in rodent populations- WHO technical reports



Recovery of plague is usually accomplished by trapping sick rodents. Recovery of dead rats with the plague may yield the organism although the low numbers may make it hard to start growth. Blood serum coated swabs help in taking the samples from tissues and inoculating medium. Full protection by inhalation and dermal exposure is critical when handling infected animals and tissues. If the carcasses are decomposed, it may be hard to grow and isolate the organism. The best means is to inoculate a sample of the decomposed tissue lesions into the nasal membranes or a shaved area of the skin of a guinea pig.

The most susceptible rodents to plague are the black house and ship rat and the less susceptible gray sewer rat which are most commonly involved. Ground and tree squirrels, prairie dogs, cats, dogs, coyotes, and bobcats have all been known to harbor the organism. The feces and urine of infected rats may also contain Y. pestis making this an alternative method of recovery. Infected soil from the burrows of rodents may also contain plague bacilli, especially prairie dog burrows as the prairie dogs have acquired antibodies to plague and are resistant to the disease itself but may be carriers.

Infections occur by breathing in air droplets from infected persons or by being bitten by rat fleas which carry the organism. Laboratory infections are easily contracted by workers who accidentally create aerosols, especially when handling tissues at necropsy of experimentally infected animals. [It is absolutely critical that gas masks or filtered air systems are used when handling cultures as this organism easily becomes airborne when the lid is removed from media containers. If it is inhaled, you will not survive. You can possibly survive the infection in cuts or bites. but you should still wear protective clothing and use disinfectants.] The Indian Rat Flea is the most effective carrier vector and is the choice to use in biological weapons.

When rats are infected heavily with the disease, their blood may contain up to 100 million cells of Y. pestis per ml. which is why fleas are so easily infected as well. In India, spraying widely with insecticides to stop malaria apparently also killed off most of the fleas and produced a drastic reduction of the incidence of plague in that country.

Another reservoir of plague is in the burrows of rodents where infected fleas may live for one to four years. These may be found in virtually every state west of the 100th meridian in the US but is most heavily concentrated in New Mexico. Where rodent populations acquire some resistance to the plague, they constitute a permanent reserve of organisms.

There is also a five year cycle of outbreaks in the United States with turns coming at the decade and mid decade marks (1990, 1995, 2000). Plague is known to be extensive in the animal reservoirs in the US and cannot be eradicated. More than half of the cases come from New Mexico each year making this the ideal location to recover the organism. Whenever a human case appears, it is estimated that about 10% of the rodents in the immediate area are infected.

The severe forms of the plague (bubonic and pneumonic) begin with the sudden onset of high fever, great prostration and varying degrees of delirium. Septicemia often follows and in the pneumonic form the mortality rates exceed 90% even with treatment.

Two other species, Y. pseudotuberculosis and Y. enterocolitica cause tuberculosis like lesions in the lymph glands and intestines respectively.

They grow well on blood and the various bile agars such as MacConkey and EMB. They grow better at closer to room temperatures of 25-30 C rather than 35-37 C. They are non-motile and non-sporing. They do not alter milk media or liquefy gelatin.

The colonies appear as smooth, gray-yellow translucent forms early on but may become rough with serrated margins with prolonged incubation which helps differentiate them.

Growth and Production

The bacteria appear as small, pleomorphic, coccobacillary gram negative forms with bi-polar staining (safety pin effect) using weak stains like methylene blue (which is a good aid in identification) under the microscope. They appear as short plump rods that measure .5-.7 um x 1.5-7.5 um, are nonmotile, nonsporogenous and are often encapsulated in fresh cultures and clinical material. Capsular material requires a stain like India ink to see.

[Bipolar staining means that the ends of the cell are heavily stained with a lightly stained area in the center when using methylene blue. This type of staining is an easy method of screening cells and colonies.]

In old cultures, pleomorphism is marked and involution or degenerative forms are very noticeable. These are markedly enlarged and stain faintly with globular, elongated or irregular forms. An old culture can actually resemble a yeast or mold. Involution can be enhanced by adding 3% sodium chloride to the media which aids in identification and isolation.

Yersinia are aerobic and facultatively anaerobic. They grow well on most ordinary media, and even those containing gentian violet and bile salts which helps isolate them. They will grow at 0 C (freezing) slowly and at about 14 C at a fair rate, and will grow up to 43 C with optimal temperature at 28 C for most species. Its pH range is 6.6 to 8.0 with 7.2-7.4 being the best.

Colonies of Y. pestis on blood agar are non-hemolytic, and at first are very small, transparent, white, and circular as discs (1 mm or less). Then they become round, opaque, and shiny and measure about 2-4 mm in 24-48 hours. Some strains are sensitive to oxygen and growth may not develop if the amount used to start the culture is small. If the inoculum is small, you can start its growth in broth or under the gelatin or agar surface. You can also add blood or sodium sulphite to the medium or simply replace the air with other gases.

In broth cultures, they form chainlike growths. If you overlay the broth with a film of oil on top, a characteristic stalactite growth occurs (as long as the growth is not shaken) which aids in identification. Much of the growth will be seen as a granular deposit at the foot and on the side of the tube or container similar to that of streptococcus.

On nutrient agar or gelatin the colonies have a delicate, drop like appearance, with a round, granular, uneven margin. When blood or hemin is added the colonies produce a darker brown color because the hemin is absorbed from the substrate.

Amino acids (proteins) are required as nitrogen sources in artificial media but vitamins are not needed. Calcium is required for cell division at 37 C but not at 26 C. Casein hydrolysate meets all requirements in liquid media and is used for vaccine and toxin production. On solid media, blood is usually added to give the best growth. Plague grows slowly on solid media and the colonies never reach a large size.

Other Yersinia species form large coccobacilli measuring .5-1 *u*m x 1-2 *u*m. They are motile at 22 C but not at 37 C which distinguishes them from Y. pestis. They are widely established in animals like rabbits, mice, and guinea pigs and have themselves caused major outbreaks of fatal septicemia in test animal populations (which means they have already been successfully tested for use as effective biological weapons). About 1,000 cases of enterocolitis caused by Y. enterocolitica were reported in the US between 1966 and 1970.

On MacConkey agar, they grow fairly well producing pinpoint lactose negative colonies that are often missed by the lab workers. These should be incubated at least 48 hours before being discarded as producing no lactose negative organisms.

Toxicity and Harm

Their are three different types of plague that can be produced in man. **Bubonic** plague is characterized by an infection of the lymph nodes which swell and form the typical buboes. **Septicemic** plague which produces small hemorrhages in the skin and mucous membranes from a generalized invasion. Pneumonic plague which spreads mostly through the lymphatic tissues of the lungs until both lungs become hemorrhagic.

In bubonic plague, the bacilli are injected into the skin of the host by the bite of an infected rat flea. The incubation period is 2-8 days after which a small number of the organisms invade the blood stream. Most of the organisms remain localized in the area lymph glands where they give rise to progressive swelling of the glands and tissues. This mass is called the primary "bubo". An intense inflammation then occurs with large numbers of Y. pestis in the bubo with hemorrhages and subsequent necrosis occurring. Secondary buboes may form in the nearby lymph nodes. The numbers of organisms in the buboes decrease and may even disappear. If the case becomes septicemic with the organism invading the blood stream, large numbers spread throughout the body and death usually follows within 10 days. The fatality rate is 60-90% in untreated cases.

In the middle ages subcutaneous hemorrhages were more prominent than those seen today and the dark spots they produced gave the disease the name "Black Death".

In septicemic plague, the organisms invade the blood directly and there is no time for involvement of the lymph nodes. This route is usually fatal making it an excellent organism for use with piercing weapons or in shrapnel devices.

Pneumonic plague begins in the bronchopneumonic areas of the lung and grow in large numbers which are seen in the sputum. This is a highly infectious form of the disease and can easily be passed from person to person much like the cold or flu which accounts for its tremendous infectivity and loss of life in the dark ages. About 21% of the bubonic plague becomes pneumonic as the organism spreads to the lungs making these carriers very dangerous to work around. Pneumonic plague outbreaks in Manchuria in 1910-1912 caused more than 60,000 deaths with 100% mortality.

There were over 13,000 cases of plague reported in Vietnam in the 1960's and since then the US government has tested Y. pestis for possible use in biological weapons with data on monkeys being sparsely reported for both bubonic and pneumonic forms.

Test animals can be infected with the prospective cultures to confirm the correct organism has been recovered. These animals are then killed and examined. In bubonic plague, the buboes are punctured with a hypodermic needle and the exudate withdrawn with a small syringe. A methylene blue or gram stained smear should reveal numerous bipolar rods. For septicemic plague, the blood is cultured and in pneumonic plague, the sputum.

A guinea pig or white rat injected subcutaneously with a fresh culture, dies within a few days and at autopsy has a marked local inflammatory condition with necrosis and edema. Related lymph glands are also involved, the spleen is enlarged and congested and has small grey-white areas in its tissues. There is also septicemia and the bacilli can be easily seen in slides of the local lesion, lymph glands, spleen, and heart blood. These conditions are also seen in rats dying of plague and it is easy to trap, recover, examine and culture from these animals with success (if you survive it).

When dead cultures are injected into animals there are marked local and toxic effects but a true exotoxin is not produced. What toxin has been produced is on the inside of the cells and is released upon lysis of the cells and has only a small role in the infectivity of the organism itself.

The plague produces an LPS toxin like the other enterobacteriacaea which is similar to that of the Salmonella endotoxin and is also antigenic for that toxin. It is toxic to lab animals, evokes a pyrogenic response in rabbits, and in purified form has an LD 50 of 1 mg in rabbits. This amount of toxin can be accounted for in infections in lab animals and for the toxic symptoms observed in human infections.

The second group of toxins produced have some of the same properties of both endotoxins and exotoxins. They are proteins but do not appear to diffuse freely into the surrounding medium like normal exotoxins do. They are released after rupture or lysis of the cell and are part of the cell membrane. These toxins have been named "murine" toxins. They are active in rats and mice but are not toxic to guinea pigs, rabbits, or chimpanzees.

When injected into mice, the murine toxins produce edema followed by necrosis and act mostly on the peripheral vascular system and liver to produce irreversible toxic and lethal effects. This toxic action is antagonized by cholera toxin and both cholera and plague should not be used together in combination weapons because of this.

The murine toxins can be prepared in highly concentrated form by ammonium sulfate fractionation and electrophoresis of extracted acetone dried bacilli. Two toxins designated A and B are recovered and have LD 50's in mice of .5-1 ug each. High titers to these toxins are observed in the serum of survivors of plague. They are produce by both virulent and avirulent strains of Y. pestis which means that noninfective strains can also be used to produce toxin weapons.

Virulence means that the organism must be able to invade first before it can cause harm. The strain can be grown and produce the harmful substance making it useful as a weapon anyway.

Plague bacilli are first dried and lysed with acetone and are then extracted with 2.5% or more sodium chloride solution saturated with toluene at room temperature for 24 hours. After centrifugation, the cells are extracted with a smaller volume of the sodium chloride solution. The combined supernatant (liquid) was dialyzed followed by concentration under vacuum drying to one third of its volume. Ammonium Sulfate 30% at a pH of 7.5 was added and left a precipitate designated fraction 1A. The ammonium sulfate was increased to 40% and the collected precipitate was designated fraction 1B and also contained the envelope antigen. The supernate from this fraction contained the toxin. The LD 50 for this fraction was .6-.8 *u*g per mouse.

Another method of concentrating the toxin is to saturate the solution with ammonium sulfate at 0 C for 24 hours to precipitate total protein and then to use the above methods to produce the desired concentration. The soluble envelope antigen may be separated from the toxin at pH of 4.7.

Toxin production in relation to cell mass is much higher when the organism s grown at 30 C than at 37 C. Toxin production peaked at 48 hours.

It is believed that both types of toxins play a direct role in the disease process but these methods are not well understood.

Some of the interesting observations of plague victims include -

1. Pregnant women infected with plague produced fetuses with no infective organisms but hemorrhages and degenerative changes that could only be caused by the plague toxin.

2. Huge levels of antibiotics given at 36-48 hours after the onset of infection produced sterile blood with no organisms but the patients still died. This also was evidence that the plague toxin causes the death and injuries rather than just the spread of the organism itself.

The combination of both the toxins and Y. pestis ability to invade unchecked make it one of the deadliest diseases known.

Plague is pathogenic to monkeys, rats, guinea pigs and other rodents as well as humans. It is an epizootic (endemic) disease among wild rats and other rodents.

Y. pestis contains two antigens, one that is somatic and heat stable, the other is heat labile at 100 C and is associated with its capsule and is formed when the culture is grown at 37 C. Vaccines used in mice based on this antigen were grown at 37 C and used in India with some success for the bubonic form. Vaccines tested for guinea pigs required the somatic antigen to be effective.

Serotypes do not occur as the Y. pestis is antigenically homogenous although more than 20 antigens are known at the present time. None of the antigens appear related to virulence. Virulence is only measured as the number of cells required to infect and kill lab animals. The most virulent strains may only require as little as 10 cells to produce an LD 50 in mice or guinea pigs.

The surface slime appearance of the colonies is due to the capsular material which is associated with the ability of the bacilli to resist phagocytosis. It is usually present in virulent strains but absent in avirulent strains. Some exceptions to this are seen in the mouse infections.

It has also been found that plague bacilli may remain viable after being consumed by neutrophils and macrophages which means that resistance to phagocytosis is an important virulence factor.

Virulent strains of plague bacilli also produce a substance called **pesticin** while other strains produce coagulase and fibrinolytic activity which always occur together and are believed to be genetically linked.

Avirulent strains have been made into virulent strains by injecting mice with the organism and adding iron with the injection. The recovered organisms become more and more virulent with each passage.

It has also been found that mutant strains that lose the ability to synthesize purines lose their virulence. They can be made virulent by adding purines in the administered doses.

There are three strains of Y. pestis that are responsible for plague.

1. Orientalis which caused the 1894 epidemic and is endemic in India, Burma, South China and the sylvatic or wild rodent plague in the western United States.

2. Antigua is the oldest strain originating in Mongolia and Manchuria and spread west with the Aryan invasions reaching the Nile and spreading through Africa. It moved back to the Mediterranean in the sixth century devastating the Roman Empire. It has since disappeared from Europe and is found isolated in Africa.

3. Mediaevalis is believed to have been mutated from Antigua and spread from the Caspian Sea throughout all of Europe causing the Black Death in the 14th centuries.

Test		Orientalis	Antigua	Mediaevalis
Acid from	Glycerol	-	+	+
	Melibiose	-	-	+
Nitrate reduction		+	+	-

New strains can be produced and modified by ultraviolet radiation, nitrogen mustard and other mutation generating methods.

Protective Measures

Y. pestis is easily killed by common disinfectants and pasteurization. Sulfadiazine appears to be as effective as the often used antiplague serum. Streptomycin, chloramphenicol, and tetracycline are also effective if administered at the onset of fever. In pneumonic plague where the infection usually begins at just one site, the use of tetracycline has had some success. Therapy must be delivered early (within the first 15 hours) and last at least ten days in order to be effective.

Rat and flea control are the most important defense during epidemics since this is the primary means of spread. The thermal death point is 55 C in 15 minutes and the organisms die quickly when subjected to drying. It is killed in sunlight, hot summer temperatures which often mitigate the spread of plague, and is killed in .5% phenol in 10-15 minutes.

Plague vaccine has been widely used for prophylactic purposes and is made growing P. pestis for four weeks at 27 C in digest broth. Phenol at .5% is added as a preservative as the culture is heat killed. Injections of 1 and 2 ml are given at 7-10 day intervals. The protective value given is modest. Living, non virulent strains seem to work a little better and the plague vaccine used for American soldiers in Vietnam seemed to give solid immunity in all cases of bubonic plague but not the pneumonic form where its effects seem short lived. Recovery from plague infection also appears to give strong immunity to subsequent infection.

Anti plague serum also affords some protection and is used by health workers for temporary protection when caring for plague victims.

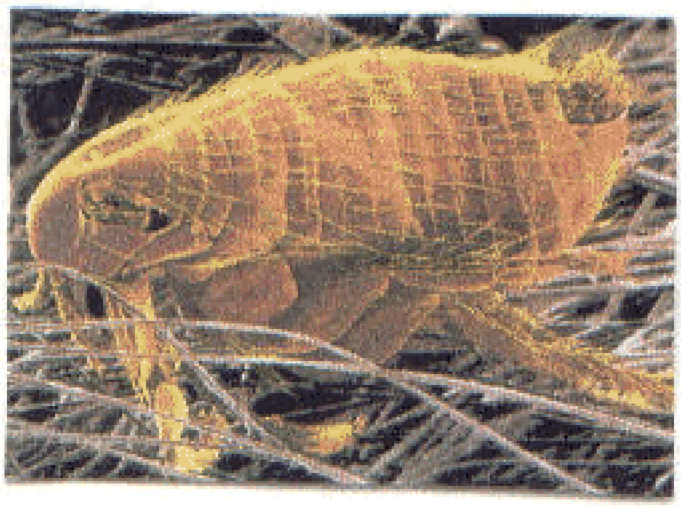
Y. enterocolitica is generally sensitive to chloramphenicol, colistin, gentamicin, kanamycin, and streptomycin. Y. pseudotuberculosis is susceptible to kanamycin, streptomycin, and tetracyclines.

The net effect is that you now have an army armed with invisible and self reproducing bullets. The only safe people in the government are those living in space suits since any vehicle, piece of mail, package, or object can be easily infected. Those in government who think they can control a population with police or an army can be easily disposed of, as well as their army. The simple idea of anthrax or plague on a tree branch with poison ivy or itching powder, or on dust inside of a sealed drawer in your home can instill fear and death in the enemy. These make easy, lethal, invisible, reproducible, and highly effective booby traps that the enemy cannot easily associate with any particular person or location. And they can be killed by the thousands just by the act of brushing against a tree branch in the wrong area.

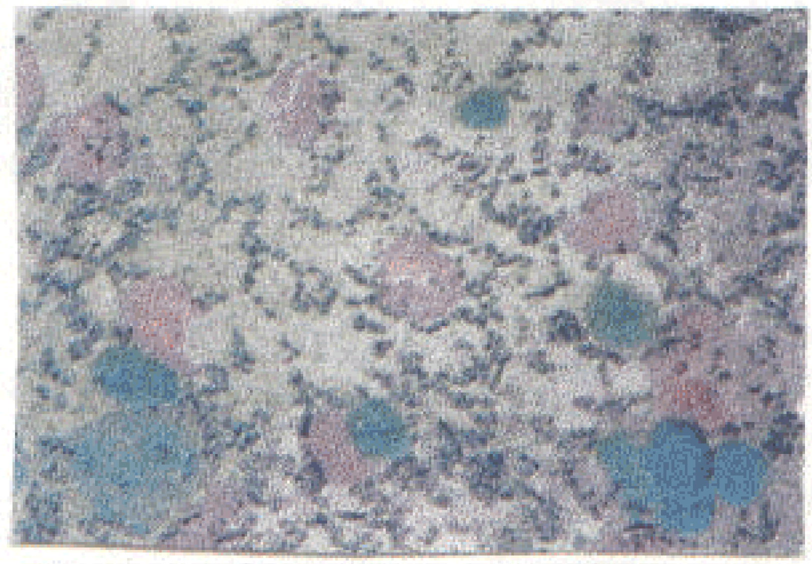
If the US government, or any government for that matter, thinks they can disarm and regulate every aspect of their citizens lives simply because they can take away their subjects money and use it to hire an armed system of police and army to enforce their rules, they are in for a rude awakening. Most people in a society follow the rules either out of fear of the law, or respect for it and their fellow man. The experiences of my life have proven over and over repeatedly that the laws of the United States (and many other countries) don't even mean anything. Even the US Bill of Rights amount to little more than a bunch of worthless words on a piece of paper. That is why I have written this book. The people of this country and not a lot of bought and paid for politicians who write "Jim Crow" financial and property ownership laws will decide under what rules we are going to live. Even the ability to raise vast sums of money and hire huge armies isn't going to protect unjust governments anymore. The only thing that can protect them now is fair laws that people can live with. If the laws of this nation are not going to be fair then it is my right, and my duty to fight this government any way I can, even if it costs me my own life. Writing this book is one way of fighting their tyranny. There are many other ways as well.

- Tim Tobiason]

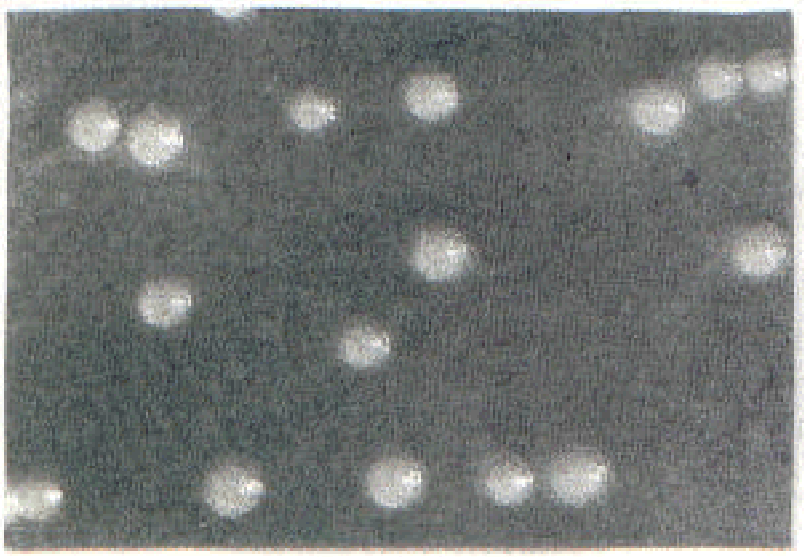
The Rat Flea "Xenoosylla cheopis"
The best vector for spread of plague.



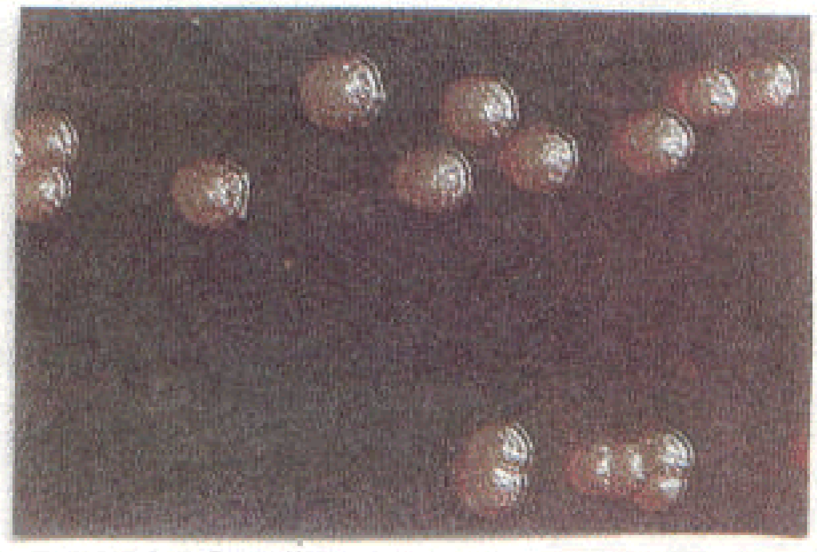
Y. pestis bacilli in a liver lesion. The bipolar staining of the cells is evident.



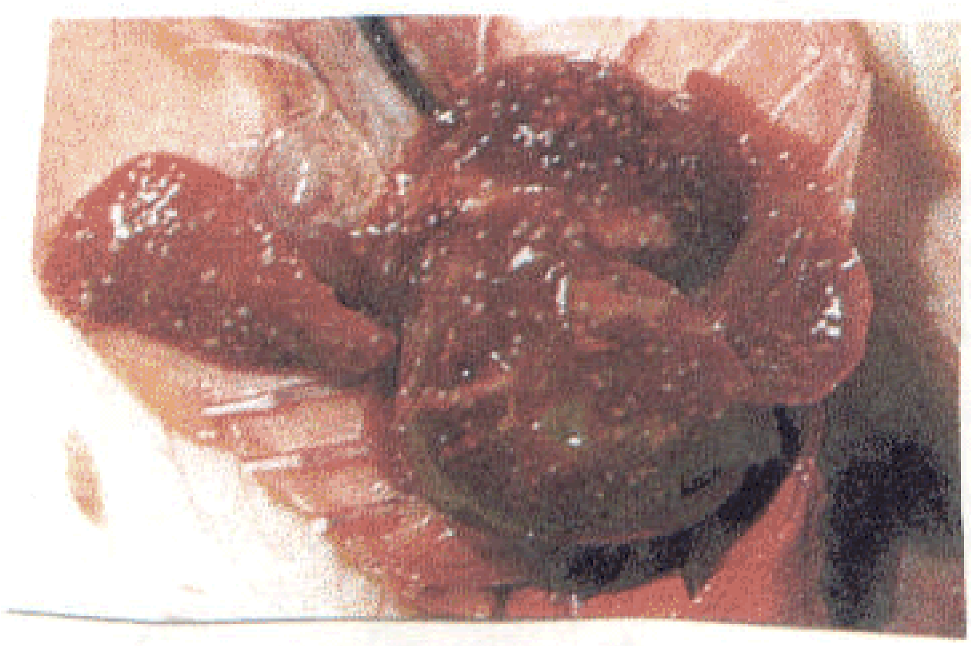
Y. pseudotuberculosis on digest agar where the colonies have a granular surface, a raised, more opaque center, and a transparent effuse edge.



Y. pseudotuberculosis on blood agar at two days. The blood agar has lysed and turned brown. They do not show the effuse edge as seen at left.



Y. pseudotuberculosis following intramuscular inoculation. Grey-white nodules are seen distributed evenly over the surface of the spleen and liver.



Comparative Toxicity of Biotoxins and Chemicals

Using strychnine as the base and calculated on a same weight basis.

Strychnine (base for comparison) =	1
Bacterial endotoxins (E Coli, etc)	.1
Fluoroacetate (C2H2O2F Na)	10
Crotactin (Snake venom, protein)	10
Sarin (Nerve gas)	15
Ricin (Plant seed)	20
Tetrodotoxin (Fish and Newt)	50
Cl. welchii Epsilon toxin	300
Staphylococcal alpha toxin	350
Diphtheria Toxin	2,000
Dysentery Neurotoxin	1,000,000
Tetanus toxin	1,000,000
Botulinum A and B	1,000,000
Botulinum D	3,000,000

Temperature Scale

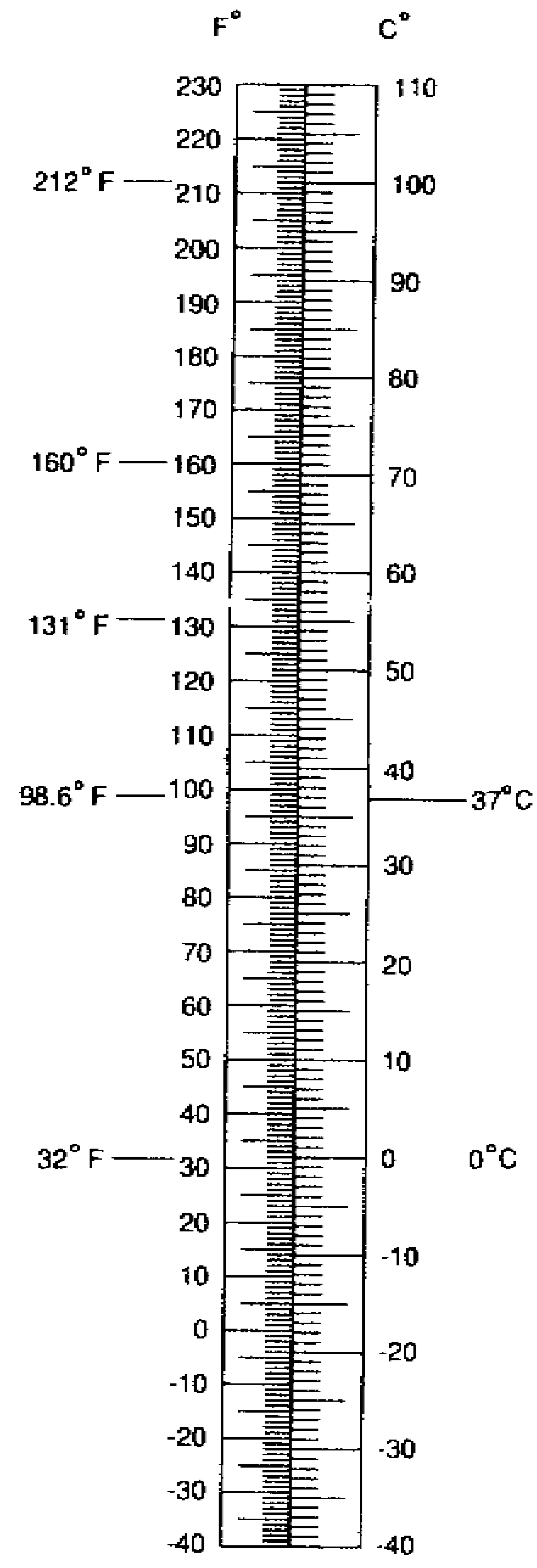
It is essential to know the correct temperatures to grow microorganisms. Most of the temperatures listed in this book are in centigrade - the numbers listed on the right side of the chart. The equivalent temperature in Fahrenheit is listed on the left side of the chart.

Body temperature = 98.6 F or 37 C

Pasteurizing milk takes place at 71 C

To convert from C = 5/9 (F - 32)
F = 9/5 (C + 32)

Water freezes at 0 C (32 F) and boils at 100 C (212 F)



Weights

One kiloton	=	1,000 kilograms (one metric ton)
One kilogram (kg)	=	1,000 grams (or 2.2 lbs)
One gram (g)	=	1,000 milligrams (or .035 ounces)
One milligram (mg)	=	1,000 micrograms
One microgram (*u*m)	=	1,000 nanograms
One nanogram (ng)	=	1/1,000 micrograms

Door Openers

A number of the bacteria we have already described produce different toxins and other substances that act as "door openers". These can have a battering ram effect where an enzyme may simply destroy the surface tissues such as a cell wall which results in the contents leaking out and the cell being destroyed, or a small part of the wall is made porous to other materials like salts that allow the free passage of these substances.

The addition of door openers to the bacteria weapons is essential to provide extra food (by turning the cell substances into unprotected food substrate) or help it gain access to underlying tissues that it can grow on and establish an infection. One good example would be the exfoliatins produced by the S. aureus which dissolve the connective tissues of the skin. This permits other organisms to invade the underlying epidermis and subcutaneous layers and in many instances become able to use the dissolved components as food.

Door openers can have a wide range of effects. One possible result of giving a live vaccine such as the anthrax, with antiserum, is that even though the antiserum stops the main toxin from causing harm, other door opening substances may allow parts of other vaccines or other chemicals and microbials to enter tissues they otherwise would not. The obvious example is the use of many vaccines, some of them live, as troops entered the Persian gulf. When these vaccines are given at home, the individual is exposed to the same microorganisms continuously and has built up some immunity which would limit the entry of these organisms while the door is being opened by the actions of the injected live vaccine organisms. If the vaccine is given when the person is introduced into a new microbial environment such as right before landing in a different country, he is now exposed to microbial populations that he has no built up immunity to. These may well enter into the opened tissues and can cause harm during or after this brief time frame.

Another possible effect is the use of door openers by themselves as weapons. The idea behind this is to use the various enzymes and proteins to open doors and allow whatever is in the environment to cause the harm. This can include the organisms already living on and in the body such as the staph and strep already described. The use of neuraminidase to break down mucus and allow diphtheria and other microbes to grow on it would be an indirect example.

When these doors are opened, the microbes in the surrounding environment as well as chemicals like organophosphate pesticides (or nerve agents), petroleum solvents, dust and gas particles and so on are all able to enter the various cells and tissues. In some cases the effects may not be observed for years like the diseases caused by the streptococcus in the last section.

In the Persian gulf war, the vaccines combined with various other agents may well have accounted for some of the syndrome symptoms observed years later. If the mycotoxins (such as the reported possible exposure to aflatoxins with their own set of door openers - produced by molds and stored by the Iraqis) were also present, whatever the troops happened to be exposed to in the area would also be absorbed into the tissues.

Preparing your own media ingredients

Sterilizing homemade cultures is accomplished simply by heating the media as follows-

Volume	Container amount held at 121 C
10 ml	20 minutes
100 ml	25 minutes
500 ml	30 minutes
1 liter	40 minutes

The larger the amount of media, the longer the sterilization time must be. Heat sensitive ingredients like blood, serum, or egg yolk must be sterilized by other means, usually using a bacterial filter.

Egg Yolk can be solidified for use in media by heating at 75-85 C in steam so that it does not dry out. The proteins completely solidify at this temperature into the form that they are held in such as a test tube or culture plate.

Agar is commercially prepared in many countries from a variety of seaweed's (Gelidium, Eucheuma, Pterocladia and others) in which the weed is dried out, extracted by hot water processes (boiling is suitable in homemade preparations), clarified, dried and then sold as a powder or as strands. Japanese and New Zealand agars produce a gel with suitable firmness at a concentration of 1-2% while other countries may require 3-4% to provide good solidification. You can also make your own agar from purchased seaweed.

Agar is prepared by mixing the powder or fiber into the liquid mixture and dissolving it in a steam heated environment (adding a pan of water in the oven) at 100 C for one hour. The result should be a clear gel which is colored only by the added ingredients. If the agar produces a color on its own, it may be hot filtered at 30 minutes to remove precipitated impurities and then the heating is completed. The ingredients can be added to the agar later as well by simply melting the agar at 45-50 C, adding the ingredients and then cooling to resolidify it.

The agar can become hydrolyzed to materials that will not solidify on cooling if acid is present during its preparation. Its starting pH should be above 5 and any acid type ingredients added after the heating process is complete.

Peptone is made by taking heart muscle, casein, fibrin, or soy flour and digesting it in a water mixture with proteolytic enzymes like pepsin, trypsin, or papain. This digestion is then filtered and the water soluble portion is then dried yielding a golden granular powder that is slightly acid with a pH of 5-7 in a 1% solution. It is hygroscopic and soon becomes sticky when exposed to air. The idea behind this as a separate useful ingredient is that it supplies water soluble peptones, proteoses, amino acids, a few inorganic salts, and other growth factors. It does not supply fermentable carbohydrates which are added separately to grow and identify specific organisms.

Casein Hydrolysate is produced by hydrolyzing the milk protein casein with hydrochloric acid and then neutralizing the mix with baking soda making it rich with salt or using a proteolytic enzyme like trypsin. This last method leaves an acid substance that needs added tryptophan to make it suitable for growing bacteria. Casein hydrolysate may be used in place of peptone in broth and other media and is useful in enriching growth mediums for many organisms.

Meat Extract is made by boiling finely ground lean beef in water which holds the unconcentrated extract. This is then filtered off (or settled and poured off) and evaporated leaving a dark, viscous paste that contains a wide range of water soluble compounds including gelatin, albumoses, peptones, proteoses, amino acids, and about 10% nitrogen compounds such as creatin, creatine, and purines. It also is rich in various vitamins, growth factors, and some carbohydrates.

Yeast Extract is produced by washing, filtering, and then heating brewers or bakers yeast to 55 C. They can also extracted with hydrochloric acid or a proteolytic enzyme. The cells are then removed from the water soluble extract by filtration and the extract is evaporated to a thick dark paste containing about 70% solids. Amino acid content is about 50% and it contains many growth factors and vitamins. It also contains carbohydrates and about 10% inorganic salts. It is used to enrich growth media.

Blood is prepared by adding sodium citrate or potassium oxalate to render it non-coagulating. It must be sterilized by filtration and not heated to avoid disintegration of the cells and denaturation of the red hemoglobin to brown derivatives. It can also be defibrinated by placing in a bottle with small glass beads and shaking continuously for 5 minutes.

Peptic digest of blood is prepared by liberating nutrients from blood cells through digesting them. The digest is prepared as follows -

Salt .85% in water	150 ml.
Defibrinated sheep's blood	50 ml
Pepsin	1 gram
Chloroform	.5 ml
hydrochloric acid	6 ml

The saline, acid, blood, and pepsin is heated in a stoppered bottle at 55 C for 2-24 hours. Add the chloroform and shake vigorously.

Serum is prepared by killing the desired animal and severing the neck artery allowing the blood to flow into a container or bottle. The clot is separated from the sides of the bottle by using a stiff wire. The bottle is kept overnight in the refrigerator and then the clear serum is pipetted off. It is also prepared from oxalated blood by storing overnight in a refrigerator which allows the corpuscles to settle. The serum or plasma is then siphoned off. It is then warmed to 37 C and calcium chloride is added (a 4% solution - 22.5 ml for each liter of serum).

Nutrient Broth is prepared by -

a) meat infusion broth made by filtering off the water from a lean meat solution mix to which peptone is added.

b) meat extract broth prepared from mixing commercially made peptone and meat extract.

c) digest broth made from the watery extract of lean meat that has been digested with a proteolytic enzyme and does not need added peptone.

Digest Broth are used to produce fast and large growth of fastidious organisms. These cultures tend to die off as they run out of food faster. The proteolytic enzyme trypsin is used and this comes from the pancreas. Papain from paw-paw may also be used.

Pancreatic extract -

Fresh pig pancreas	500 g
Water	1,500 ml
Alcohol or methylated spirit	500 ml
Concentrated hydrochloric acid	2 ml

The fat is removed form the pancreas and it is then minced and mixed with the water and alcohol. It is then shaken in a large stoppered bottle and allowed to stand for 3 days at room temperature being shaken occasionally. Filter the mix and add .1% hydrochloric acid to the filtrate. It causes a cloudy precipitate which can be filtered off although this is not necessary. The extract has a shelf life of about 2 months if refrigerated. The acid is added to the mix to retard the action of the trypsin while in storage.

Final digest broth -

Lean meat	1,500 g
Water	2500 ml
Baking soda .8% solution	2500 ml
Pancreatic extract	50 ml
Chloroform	50 ml
Hydrochloric acid	40 ml

The meat and water are mixed and heated in steam to 80 C. The baking soda mix is added and the mix is cooled to 45 C. The pancreatic mix and chloroform are then added. Incubate at 37 C for 6 hours or 45 C for 3 hours while stirring frequently. When digestion is complete, add the acid, steam at 100 C for 30 minutes and filter. The broth is stored in acid condition with .25% chloroform in a refrigerator. It should be shaken vigorously in the next 2-3 days. To use it, you adjust the pH to 8.0 with caustic soda and steam at 100 C for one hour. to precipitate phosphates. Filter while hot, adjust the pH to 7.6 and mix it into the medium.

Nutrient agar is simply nutrient broth solidified by the addition of agar. Nutrient agar is often simply called "agar".

Semi solid agar is when a small concentration of agar is added (.2-.5%) so that they do not solidify. This allows motile strains to rapidly spread across the medium while non-motile strains do not spread. At .05-.1%, it prevents convection currents and retards the diffusion of air into the media for the growth of anaerobic organisms in liquid.

Concentrated agar is made by adding 4-8% agar and is used to prevent rapid spreading causing motile to form discreet colonies. It takes longer to dissolve and cool and is more difficult to handle than ordinary agar.

Peptone water is used to make sugar fermentation media. It is prepared by mixing -

Peptone	10 g
Sodium chloride	5 g
Water	1 liter

Blood agar is made by mixing 5-10% blood to sterile nutrient agar that has been melted and cooled to 55 C. If the blood concentration is 10% or more the agar is made in two layers with the top layer being very thin. If nutrient broth is added it becomes an enriched blood medium.

Heated (Chocolate) blood agar is used for growing haemophilus and other fastidious organisms and is made by heating 10% sterile blood in sterile nutrient agar at 75 C which melts the agar. They are mixed with gentle agitation until the blood becomes a chocolate brown in color (usually about 10 minutes). This ruptures the red blood cells and nutrients are then liberated into the liquid. The agar is then poured as plated or into tubes.

It may also be made by simply heating blood agar plates at 55 C for 1-2 hours in a hot air oven. H. influenza grow larger colonies on media prepared this way.

Milk agar is made by mixing 100 ml of fresh milk with 200 ml of nutrient agar with 3% agar added. The milk is heated to 60 C, is shaken, and then sterilized at 121 C for 20 minutes. The agar is melted and then cooled to 56 C and then they are mixed and poured. This media grows staph extremely well.